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CLINICAL LABORATORY METHODS AND DIAGNOSIS

FRANKEL AND REITMAN, EDITORS

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Volume 2 GRADWOHL'S

CLINICAL LABORATORY METHODS AND DIAGNOSIS

A textbook on laboratory procedures and their interpretation

Edited by

SAM FRANKEL, Ph.D.

Director of the Division of Biochemistry, Department of Laboratories,
The Jewish Hospital of St. Louis, St. Louis, Mo.; Member, American Association of
Clinical Chemists and American Chemical Society

STANLEY REITMAN, M.D.

Director of the Gradwohl Laboratories and the Gradwohl School of
Laboratory Technique, St. Louis, Mo.; Member, Teaching Staff,
Department of Medicine, The Jewish Hospital of St. Louis, St. Louis, Mo.; Editor-in-chief,
The Laboratory Digest; Member, American Public Health Association, Royal Society
of Health (London), American Society for Microbiology, and American
Society of Tropical Medicine and Hygiene

Associate Editor of Microbiology

ALEX C. SONNENWIRTH, Ph.D.

Director, Division of Microbiology, Department of Laboratories, The Jewish Hospital of
St. Louis, St. Louis, Mo.; Assistant Professor, Department of Microbiology,
Washington University School of Medicine, St. Louis, Mo.; Member, American Society for
Microbiology, American Association of Blood Banks, and American Association
for the Advancement of Science

SIXTH EDITION

Volume 2 GRADWOHL'S

Rutherford Birchard Hay

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1877
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Figures 330 to 794, including 34 in color

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SIXTH EDITION

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Contributors

PHILIP G. ACKERMANN, Ph.D. Protein-bound iodine; blood lipids
Research Assistant Professor, Division of Gerontology, Washington University School of Medicine, St. Louis, Mo.; Chief Biochemist, St. Louis Chronic Hospital, St. Louis, Mo.

JOHN D. BAUER, M.D. Hematology; spinal fluid
Director of Laboratories, DePaul Hospital and Faith Hospital, St. Louis, Mo.; Assistant Professor in Pathology, Washington University School of Medicine, St. Louis, Mo.

EVERETT S. BENEKE, Ph.D. Medical mycology
Professor in Mycology, Biology Research Center, Michigan State University, East Lansing, Mich.

M. R. CASTAÑEDA, M.D. Brucella
Director, Investigaciones Medicas, Hospital General, Mexico, D. F.

W. HAROLD CIVIN, M.D. Model of a clinical laboratory
Director of Laboratories, The Queen's Hospital, Honolulu, Hawaii

NORMAN E. DEWAR, Ph.D. Disinfectants, sanitizers, and antiseptics
Director of Research, Vestal Laboratories Inc., St. Louis, Mo.

JULIUS ELSON, M.D., F.A.C.P., F.A.C.C. Electrocardiography
Instructor in Clinical Medicine, Washington University School of Medicine, St. Louis, Mo.; Assistant Physician, Barnes Hospital, St. Louis, Mo.; Attending Physician, Teaching section of Medical Staff, The Jewish Hospital of St. Louis, St. Louis, Mo.; Chief, Department of Medicine, Christian Hospital, St. Louis, Mo.; Cardiologist to Christian Hospital, St. Louis, Mo.

ADDINE G. ERSKINE Blood groups
Principal, Gradwohl School of Laboratory Technique, St. Louis, Mo.; Assistant Editor, The Laboratory Digest

SAM FRANKEL, Ph.D. Clinical chemistry; urinalysis; gastric secretion and duodenal content
Director, Division of Biochemistry, Department of Laboratories, The Jewish Hospital of St. Louis, St. Louis, Mo.

M. L. GRAY, Ph.D. Listeria monocytogenes
Associate Bacteriologist, Veterinary Research Laboratory, Montana State College, Bozeman, Mont.

LEWIS J. GRIFFITH, Ph.D. Bacterial sensitivity testing

Acting Chief, Clinical Laboratories, and Chief, Staphylococcus Reference Laboratory, Veterans Administration Hospital, Batavia, N. Y.; Instructor in Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

MICHAEL H. IVEY, Ph.D. Parasitology

Assistant Professor, Department of Microbiology, University of Missouri School of Medicine, Columbia, Mo.

J. MEHSEN JOSEPH, Ph.D. Viral and rickettsial diagnostic procedures

Chief, Division of Virology, Bioanalysis and Training, Bureau of Laboratories, Maryland Department of Health, Baltimore, Md.; Instructor in Microbiology, University of Maryland, Baltimore, Md.

SIDNEY KAYE, Ph.D. Toxicology

Professor of Toxicology, Department of Pathology, School of Medicine, San Juan, Puerto Rico; formerly Toxicologist, Office of the Chief Medical Examiner, Commonwealth of Virginia, and Director, Poison Control Centers, Richmond, Va.

GERALD KESSLER, Ph.D. An automated system of analysis

Supervising Biochemist, Division of Neoplastic Medicine and Clinical Research Center, Montefiore Hospital, New York, N. Y.

TAKESHI KODAMA, M.D. Streptococci

Director, Kanagawa Prefectural Public Health Laboratory, and Chairman, Japanese Association of Public Health Laboratories, Yokohama, Japan

†ARAM A. KRAJIAN, Sc.D. Tissue cutting and staining

Formerly of Department of Histopathology, Los Angeles County Hospital, Los Angeles, Calif.

HARRY W. MARGRAF Hormone assay in clinical diagnosis

Research Instructor, Washington University School of Medicine, St. Louis, Mo.

SACHIMOHAN MUKERJEE, M.B., D.Phil. Bacteriophage

Head, Division of Microbiology, Indian Institute for Biochemistry and Experimental Medicine, Calcutta, India.

ERWIN NETER, M.D. Bacterial hemagglutination

Director of Bacteriology, Children's Hospital, Buffalo, N. Y.; Associate Professor of Bacteriology and Pediatrics, State University of New York at Buffalo Medical School, and Research Professor of Microbiology, State University of New York at Buffalo Graduate School, Roswell Park Division, Buffalo, N. Y.

†G. PANJA, D.Bact.(Lond.), F.N.I., F.D.S.(Lond.) Vibrio comma

Professor of Bacteriology and Pathology (retired), School of Tropical Medicine, Calcutta, India.

†Deceased.

SAMUEL L. PHARR, M.S. Cytology

Research Cytologist, Department of Medicine, University of California School of Medicine, San Francisco, Calif.; San Francisco General Hospital, San Francisco, Calif.

JOHN P. RANSOM, Ph.D. General methods in bacteriology

New England Institute for Medical Research, Ridgefield, Conn.

STANLEY REITMAN, M.D. The microscope; basal metabolism; gastric secretion and duodenal content; feces

Medical Director, Gradwohl School of Laboratory Technique, St. Louis, Mo.; Editor-in-Chief, The Laboratory Digest; Teaching Staff, The Jewish Hospital of St. Louis, St. Louis, Mo.

ALVIN L. ROGERS, B.S. Medical mycology

Research Associate, Biology Research Center, Michigan State University, East Lansing, Mich.

A. CHARLOTTE RUYS, M.D. Leptospira

Professor in Bacteriology, Immunology, and Epidemiology, University of Amsterdam, The Netherlands.

ALEX C. SONNENWIRTH, Ph.D. Clinical bacteriology; serology

Director, Division of Microbiology, Department of Laboratories, The Jewish Hospital of St. Louis, St. Louis, Mo.; Assistant Professor, Department of Microbiology, Washington University School of Medicine, St. Louis, Mo.

MAURICE S. TARSHIS, Ph.D., F.A.P.H.A. Mycobacteria; nocardia

Director, Tuberculosis Research, Medical Research Laboratory, Veterans Administration Hospital, Alexandria, La.; Member, Committee on Pathology and Microbiology, American College of Chest Physicians

GELSON TORO, Ph.D. Protein-bound iodine; blood lipids

Assistant Director of Scientific Research, The Gerontological Research Foundation, St. Louis, Mo.; Consultant in Biochemistry, St. Louis Chronic Hospital and Missouri Clinical and Biochemical Laboratory, St. Louis, Mo.

In memoriam

Rutherford Birchard Hayes Gradwohl, M.D., Sc.D.

MARCH 4, 1877—MAY 9, 1959

May 9, 1959, marked the end of a professional life of sixty years devoted to the practice of medicine and to its advancement as a science. Dr. Gradwohl's career reflected a diversity of interests, the eagerness to investigate new fields of knowledge, and the spirit of inquiry and enthusiasm that marked him as a person. Born in Baltimore, Maryland, the seventh son of a seventh son, Dr. Gradwohl was educated in the public schools and in 1898 was graduated in medicine from Washington University School of Medicine, St. Louis, Missouri, later serving his internship at St. Louis City Hospital. He began the first of a series of postgraduate studies in Europe in 1900 where, under the guidance of Professor Langerhans and Dr. Rudolf Virchow, he entered the field of pathology, which was to become the major interest of his life. He studied for a year in Paris at the Pasteur Institute, returning home to accept a position as Professor of Bacteriology and Pathology at the Marion Sims–Beaumont Medical School, later to become the St. Louis University School of Medicine.

While serving as autopsy physician for the coroner of the City of St. Louis, he became interested in legal medicine and later, in 1934, along with Captain Elias Hoagland, was instrumental in establishing the Crime Laboratory of the St. Louis Police Department. This interest in legal medicine led, at the age of 72 years, to his founding and becoming the first president of the American Academy of Forensic Sciences, which held its first meeting in Chicago on January 26-28, 1949. From the establishment of the Crime Laboratory at the St. Louis Police Department until his death, Dr. Gradwohl was director of that laboratory. Under his guidance, it became one of the most complete police laboratories in the country.

Clinical Laboratory Methods and Diagnosis became a major contribution to the literature of the laboratory when it appeared in its first edition in 1936 as a one-volume text. During Dr. Gradwohl's lifetime, the textbook appeared in five editions. Many of the present contributors had collaborated with him in his writings. In addition to this text, Dr. Gradwohl wrote, in 1917, what was probably the first book in the English language on blood and urine chemistry and later, in 1932, published his own book entitled *Laboratory Technique,* assisted by his wife, Ida Emmons Gradwohl, and his niece, Addine Gradwohl. In 1929 he translated Schilling's *Das Blutbild (The Blood Picture)* from German into English. He is known also for his textbooks *Clinical Tropical Medicine* and *Legal Medicine.*

Dr. Gradwohl became interested in the work of Landsteiner and Wiener on the Rh factor and in 1939 and 1940 studied under their direction, introducing their work in his many monographs and lectures. In 1948 he enrolled at Cornell University to study exfoliative cytology under Dr. George Papanicolaou and introduced the latter's test in St. Louis and elsewhere.

In 1937 he established *The Laboratory Digest,* a monthly review of clinical laboratory literature, which has continued publication since that time.

A Commander in the United States Naval Reserve, he served first as Lieutenant Commander in 1917, assembling a Navy Hospital Unit in St. Louis consisting of a surgeon, an internist, a radiologist, a laboratory technologist, and an oculist. He was later transferred to the Naval Hospital and Training Station in New Orleans, Louisiana. He served in the Reserve until his retirement in 1940. One of his first suggestions to the Surgeon General was made in about 1932, urging the recruitment of a highly trained specialist reserve corps, with units in each medical center. Early in the 1930's he had suggested to the Surgeon General that a radical change be made in personnel records, namely, that the blood group and type of each individual be tested and the results included on health records and identification disks. This was adopted and is still in effect.

With a knowledge of German, French, Spanish, and Italian, as well as English, Dr. Gradwohl was able to utilize much of the technical medical literature of the Western World, and this information was passed on to his students at the school he had founded in 1920, as well as to those who used his textbooks and read his monographs.

He was a Fellow of the Royal Society of Tropical Medicine and Hygiene (London), a Member of the Royal Society of Health (London), a Fellow of the American Public Health Association, a Fellow of the American Urological Association, and for many years a Fellow of the American Medical Association. He became an honor member of the Sociedad Cubana de Policiologia y Criminalistica in 1949 and others too numerous to mention. His last two honors came in 1958, when he was unanimously elected foreign corresponding member of the Society of Legal Medicine and Criminology of France and the German Association of Legal Medicine.

Dr. Gradwohl was Pathologist to Christian Hospital in St. Louis from October, 1925, to the date of his death. He was Pathologist to the St. Louis County Hospital from October, 1933, to April, 1937. He has been referred to as the "dean of American legal medicine" and the "father of forensic pathology" in this country. In 1954 he received an award from the American Academy of Forensic Sciences for his outstanding work in legal medicine.

With his many achievements in numerous fields, Dr. Gradwohl will always be remembered for his work in the clinical laboratory, primarily as a teacher of laboratory methods. As Dr. Alexander S. Wiener has stated, "Because of his encyclopedic *Clinical Laboratory Methods,* he has left a permanent heritage in those workers who have used his books and who have studied in his school. As Founder of the American Academy of Forensic Sciences he has also made an invaluable and permanent contribution to that field."*

A. G. E.

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*The Laboratory Digest, Special Issue, June, 1959.

With love to
Dodo, Marsha, and Scott Frankel
Helene, Laurie, and Laurence Reitman
and
Rosaline, Maurice, and Betty Sonnenwirth
we dedicate this book

Preface

The sixth edition of *Gradwohl's Clinical Laboratory Methods and Diagnosis* has been essentially rewritten, but Dr. Gradwohl's original purpose, that this work serve as a reference source, textbook, and laboratory manual, has been retained. We have therefore included general discussions, detailed instructions of procedures, and information pertinent to the interpretation of results, as well as an extensive bibliography.

The contributions of twenty-nine individuals have permitted the editors to obtain authoritative coverage of the vast field of laboratory medicine. There was surprising agreement among the experts concerning topics common to two or more fields. When, however, different schools of thought were represented, we attempted to adequately present both points of view.

The section on Clinical Chemistry has been arranged so that each chapter contains material according to category. It is impossible, of course, to have a system that completely avoids overlapping material. A typical example is the test for alkaline phosphatase activity, which is considered to be of value in the investigation of liver function but which also falls into the category of enzymes.

The entire discussion on colorimetry has been drastically shortened and emphasis has been shifted to calibration technics. Where visual colorimetry is described as the means for assay, conversion calculations are shown so that the procedure may be transferred to the photoelectric colorimeter with little or no change in methodology. This is done as a compromise so that those laboratories that are still using a visual colorimeter, and there are many, may either continue to use the procedure or, if desired, convert to the more modern method.

A good deal of material from the fifth edition has been omitted to make available the space required for new material. The decision as to which of the procedures to delete, however, was a most difficult problem. Two sources of information were used in evaluating the requirements of laboratories: data from a survey sent to several hundred laboratories plus an examination of the records of chemistry tests performed at The Jewish Hospital of St. Louis.

The results of the survey were most interesting and yielded considerable information pertinent to this problem of deciding which material to retain, delete, expand, or shorten. One example of the manner in which the information was used may be seen from the results concerning methods for performing urea nitrogen assays. Only 2.8% of the laboratories responding to the survey used the aeration method, compared with 75% using urease followed by direct nesslerization and 16% using diacetylmonoxime. The critical need for space was such that the relatively large discussion on the aeration method had to be omitted. Other deletions were decided in a similar manner.

In certain cases, procedures were exchanged. A typical example of this is found in the discussion of flame photometry. This edition describes the procedure employing the Coleman Flame Photometer which is used in 84% of the laboratories responding to the survey, whereas only 11% were using the Beckman method described in the fifth edition.

Several procedures, either new or new to this edition, are included, as well as modifications of many standard methods. This again was decided partly on the basis of the survey results. The discussion of paper electrophoresis was considerably expanded. Two additional glucose assays, both yielding "true glucose" results, have been added. Two methods for uric acid, one new and one not so new, have been included. Enzymology has been greatly expanded, consistent with the current trend. In addition, several tests have been incorporated for the assay of serum iron, iron-binding capacity, tubular reabsorption of phosphorus, sweat chlorides, and many others to bring the text up to date.

One more important factor must be considered. Although it may appear incongruous to retain old material at the expense of excluding new procedures, a little reflection on the matter will justify this decision. Procedures that have been used for several years, the weaknesses having been corrected by numerous modifications during extensive experience, are known to all as to their usefulness and their limitations. The new ones, however, have not yet stood the test of time and are still of unknown value. It is for this reason that some of the old classical material remains, perhaps slightly modified, but essentially unchanged.

Perhaps the most significant advances have been in the area of lipids, protein-bound iodine, steroids, and automation, all of which have become extremely important in modern clinical chemistry. In each case, these subjects have been treated by authorities in the field.

Protein-bound iodine has been completely rewritten by investigators who have had an enormous amount of experience in this area (Toro and Ackermann). All acceptable procedures for the assay of protein-bound iodine, including comments concerning advantages and disadvantages, have been included.

The same contributors have submitted the material on lipids, including cholesterol, a field that has been the basis of their research for many years.

Hormone assays have been compiled into a single chapter which includes certain of the pregnancy tests (Margraf).

The chapter on automation (Kessler), impossible to include in detail in a reasonable amount of space, nevertheless offers a sound introduction to an area of laboratory technic which is undoubtedly one of the focal points of all future progress in the clinical laboratory.

The section on Toxicology (Kaye) has been thoroughly rewritten so that emphasis has been placed on the most modern methods. Included are discussions of tests and actual cases which exemplify the information presented in the text. This is, without question, the most complete collection of data on toxicology to be found in any general laboratory text.

Part III, Microbiology, was almost completely rewritten, reflecting the considerable array of changes in methodology, nomenclature, and materials that have taken place since the last edition of this book. It now consists of five sections: Bacteriology, Serology, Viral and Rickettsial Diagnostic Procedures, Medical Parasitology, and Medical Mycology.

The Bacteriology section is the result of the efforts of several contributors (some of whom were originally recruited by Dr. Oscar Felsenfeld).

The nomenclature and classification of bacteria used here, with some exceptions indicated in the text, are based on the seventh edition of *Bergey's Manual of Determinative Bacteriology*. The methods and procedures selected and described, new and old, are those that proved useful and reliable in the hands of many competent workers and which, in some cases, were originated by the contributors. In addition, alternate methods are described, which, where applicable, allow for flexibility with regards to available manpower, materials, and laboratory space.

The chapters on special methods, stains and staining procedures, and media, tests, and reagents (Sonnenwirth) include considerable new material on anaerobic technics, fluorescent antibody methods, maintenance of cultures, vaccine preparation, many useful newer media and, wherever available, recommended standard methods for staining and physiologic and biochemical tests. The chapter on collection and culture of specimens and guides for bacterial identification (Sonnenwirth) encompasses recommended procedures for the collection and examination of specimens and details of the media and methods to be used for primary cultures of specimens from various sources. It also includes the examination of primary cultures and describes, with the aid of various guides, preliminary steps for the identification of various bacteria.

A large number of newer methods were incorporated in the procedures for identification, such as the bacitracin and fluorescent antibody technics for group A streptococci, the use of Tinsdale medium for *Corynebacterium diphtheriae* and *Corynebacterium ulcerans*, and the methods recommended by the Communicable Disease Center, United States Public Health Service, for cultivation and identification of *Neisseria gonorrhoeae*. New technics for differentiation of *Actinomyces* and for identification of clostridia are also included.

A new feature is the outstanding discussion on *Listeria monocytogenes* (Gray), the important role of which is increasingly recognized in both human and animal infections.

The detailed and up-to-date contribution on mycobacteria (Tarshis) is, in the opinion of the editors, without parallel among contemporary textbooks. In particular, the discussion of unclassified mycobacteria and of the methods for determination of antituberculosis drugs in body fluids fulfills an important need.

The discussions of *Brucella* (Castañeda) and of *Vibrio comma* (Panja) were contributed by leading experts on the subject. New to this edition are detailed discussions on *Pseudomonas, Aeromonas, Serratia, Chromobacterium, Alcaligenes, Flavobacterium, Mimeae, B. anitratum, Spirillum,* and *Bacteroides* (Sonnenwirth).

The discussion of Enterobacteriaceae was completely revise and it now incorporates the new taxonomic schema and methods of P. R. Edwards and W. H. Ewing (Communicable Disease Center, United States Public Health Service). The sections on *Leptospira* and PPLO were completely revised and updated (Ruys). Dark-field microscopy, as used and recommended by the Venereal Disease Research Laboratory, Venereal Disease Branch, Communicable Disease Center, is incorporated in the material on *Treponema.*

Bacterial sensitivity testing is discussed in a completely new contribution (Griffith) which encompasses a description and critical evaluation of all presently used technics. Sanitary bacteriology now includes new procedures for the testing of disinfectants, sanitizers, and antiseptics (Dewar) and some technics useful in the prevention and investigation of hospital-acquired infections.

In view of the numerous advances in the serologic diagnosis of both infectious and other diseases, the section on Serology was considerably revised and enlarged. In the chapter on tests for syphilis, some older tests were eliminated, while the Reiter protein complement fixation test (RPCF), the fluorescent treponemal antibody test (FTA), and the rapid reagin tests (RPR and USR) were added. A unique contribution is the chapter on bacterial hemagglutination (Neter), not heretofore available in a textbook of this nature. The chapter on miscellaneous serologic tests describes the newer technics of gel diffusion, latex, and bentonite tests and includes discussions of serologic tests employed in the diagnosis of rheumatoid arthritis, the detection of thyroid antibody, and the lupus factor.

The great strides made in the laboratory diagnosis of viral diseases have been amply covered in the six chapters contained in the section on Viral and Rickettsial Diagnostic Procedures (Joseph).

In the chapter on classification and general consideration of viruses, considerable material on newer viruses, such as hemadsorption viruses, Sendai virus, Coe, JH, and 2060 viruses, reovirus, as well as newly described enteroviruses, is presented. A wealth of detailed information on technics is furnished in the chapter on tissue culture and chick embryo methods. Preparation of glassware, formulas of growth and maintenance media, and details for establishing continuous cell cultures are included. Detailed instructions for handling of specimens, the viral spectrum of host tissues, and various tests for typing (serum neutralization, hemadsorption) are described in the chapter on routine procedures for isolation and identification of viruses.

The serologic diagnosis of viral infections is described in great detail in a separate chapter. A most valuable addition is the chapter on cytologic and cytochemical technics for the study of viral infections. The last chapter of this section presents a review of rickettsiae and rickettsial diseases and describes methods for isolation and identification of rickettsiae and for serologic diagnosis of rickettsial diseases.

The section on Medical Parasitology has been completely rewritten (Ivey). Following a review of classification of animal parasites, methods of transmission and host-parasite relationships are discussed. Both protozoa and helminths are treated in detail, with geographic distribution, epidemiology, morphology, and laboratory diagnosis presented for each parasite.

Of invaluable aid will be the chapter on laboratory procedures in which both routine and specialized technical procedures are presented, including considerable new material. All reagents and solutions used are listed with instructions for preparation, followed by a description of technics for preservation, staining, and concentration of fecal specimens.

Among the new additions are the Valet, Weinstein, and Otto vital stain, the MIF fixative and stain, a modification of the Ritchie method, and the MIFC method. New details are described regarding the preservation and mounting of helminth adults and eggs. The section on cultivation methods of parasites has been enlarged.

The discussion of serologic and immunologic procedures includes a most complete description of recent procedures, such as the bentonite flocculation and latex agglutination tests for trichinosis, the Sabin-Feldman dye test for toxoplasmosis, and several others.

The section on Medical Mycology has been revised and updated (Beneke and Rogers). A concise chapter is devoted to the characteristics of true fungi, including structural details, modes of reproduction, and classification. The chapter on technics for the study of pathogenic fungi includes such newer procedures as the acridine orange fluorescent stain and the description of a slide culture method.

The information supplied in the chapter on mycoses includes, among others, newer media such as Trichophyton agars and growth patterns for the various species of *Trichophyton,* newly described species of *Microsporum,* and additional technics for the identification of *Candida albicans.* In addition, immunologic and serologic procedures applicable to the diagnosis of mycoses are discussed.

The section on Hematology (Bauer) contains a comprehensive treatment of this subject, including the latest material from the German literature as well as the American and English. The technics described have all been well tried and are presented in a step-by-step manner to increase the usefulness of the section. As in previous editions, much space is devoted to the description of the morphology and physiology of the cellular elements, enriched by remarks of their appearance in phase microscopy and electron microscopy.

A discussion dealing with human chromosomes (peripheral blood culture method) and sex chromosomes (peripheral blood smear method) is included.

In the material on the anemias, not only is the erythrokinetic approach stressed but also the biochemical and enzymatic defects, as well as the nonhematologic findings, such as aminoaciduria and bone and skin changes. Mention is made of vitamin B deficiency in blood diseases, of glucose-6-phosphatase activity and haptoglobins in hemolytic anemias, and of the amino acid pattern of hemoglobins. The discussion on macrocytic anemias includes the intermediate megaloblasts and mentions the determination of formiminoglutamic acid in folic acid deficiency as well as rare biochemical causes of macrocytic anemias, such as orotic acid excretion and inhibition of thymine synthesis.

The classification of porphyrias is based on clinical as well as biochemical criteria.

Discussions are included on such newer concepts as sideroachrestic anemias, myeloproliferative disorders, and functional platelet disorders.

The discussion of coagulation defects suggests a practical approach to their investigation by starting with simple basic tests, working up to more complex diagnostic procedures.

A chapter on the hematology of infants and children will be of particular value to the pediatrician and general practitioner. This special aspect of the subject is too frequently ignored.

The data from the fifth edition pertaining to normal blood findings in domesticated animals has been retained. This information is not usually found in a text of laboratory medicine, and its inclusion is another example of the completeness of this section.

The summary of changes in blood cellular elements and hemoglobin in various diseases is extremely valuable, presenting a mass of information in a concise, readily available form.

One of the outstanding contributions to the sixth edition is that on exfoliative cytology (Pharr). One of the most recent additions to the clinical laboratory, it has become an extremely valuable force in efforts to combat neoplastic diseases. This section gives the reader an excellent fundamental concept of the field and the specific technics employed.

The section on Blood Groups, Transfusion, and Medicolegal Applications (Erskine) has been completely rewritten. It has been critically read by both Dr. Lester J. Unger and Dr. Alexander S. Wiener, and their suggestions and corrections have been incorporated in the text. It is now probably the most complete discussion of the subject to appear in any general textbook on laboratory methods. The exact details of all methods of identifying blood factors and agglutinins are given, and these include the factors and antibodies in all the blood systems known at the time this section was prepared for publication.

A short résumé on the only known sex-linked blood group factor has been included to bring the material up to date. The International Nomenclature, as suggested by Wiener, is followed throughout, with a recapitulation of the English system of nomenclature. As suggested by the Committee on Medicolegal Problems of the American Medical Association, agglutinogens and phenotypes are printed in lightface type, blood factors and their corresponding antibodies in boldface, and genes and genotypes in italics. The latest approved methods of identification of common and rare blood factors are included, as are technics of cross-matching, posttransfusion reaction "work-up," isoimmunization, the nature of antibodies, tests for agglutinins in blood serum, titrations, and the "cross-reacting antibody" or anti-C of group O serum.

Other subject matter includes erythroblastosis fetalis, exchange transfusions, preparation of blood for transfusion, small units of blood, extracorporeal circulation, preparation of packed red blood cells, the choice of blood donors, untoward reactions to blood transfusions, the technic of whole blood transfusion, preservation of whole blood in the frozen state, the American Red Cross blood program, differential agglutination, prevention of hemolytic reactions, blood plasma as a substitute for whole blood in transfusion, plasma and serum fractions, platelet transfusions, pyrogens, dried plasma, the medicolegal aspects of blood grouping, including tests for exclusion of paternity, blood groups and heredity, blood groups and disputed maternity, the identification of blood stains, medicolegal aspects of blood transfusions, infectious diseases transmitted by transfusion, and other accidents following transfusion.

The section on Tissue Cutting and Staining (Krajian) has been extensively revised. Dr. Krajian, unfortunately, died in May, 1962, during the time the sixth edition was in press.

The section is profusely illustrated with original Krajian photomicrographs, some in black and white and some in color. New material includes otohistologic technic—advances in otohistologic selective stains and chemical coloration methods by the use of radioactive metallic salts and other rare metallic salts and chemicals; a new selective hematoxylin stain demonstrating smooth and striated muscle fibers, elastic and connective tissue, bone tissue, nerve fibers, plasma cells, erythrocytes, and leukocytes; a new differential nerve stain demonstrating fibrin, smooth and striated muscle fibers, elastic and connective tissue, bone tissue, hair cells, erythrocytes, and leukocytes; chemical coloration of temporal bone sections for the identification of bone tissue, smooth and striated muscle fibers, connective tissue, and nerve fibers; a new chemical coloration method for the demonstration of nerve fibers, smooth and striated muscle fibers, connective tissue, bone tissue, blood vessels, bone marrow, and other elements; a myelin sheath stain applicable to frozen sections from central nervous system, both human and animal; and a new decalcification method on temporal bones, employing a controlled shaking machine and modified decalcifying fluid.

This section could almost be considered a textbook in itself, so complete is it in its discussions of histologic methods.

Electrocardiography (Elson) includes not only an excellent discussion of the fundamentals but also information that is necessary for interpretation. The normal electrocardiogram and the more common abnormalities are discussed in detail.

Once again the index has been expertly prepared by Mrs. Addine Erskine, and the extensive use of cross references will enable the reader to locate any desired material rapidly and without frustration. It is only through the serious effort and the excellent cooperation of all the contributors that the preparation of this text was possible. We wish to express to them our sincere thanks and indebtedness.

Sam Frankel
Stanley Reitman
Alex C. Sonnenwirth

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CLINICAL LABORATORY METHODS AND DIAGNOSIS

A textbook on laboratory procedures and their interpretation

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Section One

HEMATOLOGY

Hematology is an important department of the laboratory. For many years hematologists have been concerned with various hematologic procedures: first, with an examination of unstained blood and later with the use of specific tinctorial methods for the demonstration of various kinds of white cells. Then methods of absolute blood counting were introduced, together with special tests, such as blood sedimentation, cell volume, bleeding time, clotting time, blood typing, oxydase reaction, supravital staining, and a method of differential counting introduced by Victor Schilling.

Although technical advances have been made in blood examinations and many new clinical observations have been recorded regarding the various blood dyscrasias, much of our information in the past has come from anatomic investigations.

Recently, however, more important advances have been made since the physiologists and experimental physiologists have together applied themselves to the unknown problems of blood development and its various aberrations. Within the past 15 yr. immunohematology has become a branch in itself. The investigators in this field have added much to our knowledge of blood dyscrasias. The use of anticoagulants in the treatment of thromboembolic disease has stimulated a renewal of interest in the mechanism of blood coagulation. Pauling's work in hemoglobin has opened an entirely new field in molecular disease of hemoglobin. These are but a few examples of the dynamic approach to problems in the field of hematology.

In connection with the contents of this section, one must bear in mind that hematology is not a single science but rather a part of clinical medicine. A good hematologist must first and foremost be a skilled clinician capable of using all the diagnostic methods known to medicine necessary to arrive at the correct hematologic diagnosis. This point has been emphasized by Riser[1] in an article entitled, *The practical handling of hematology by the clinical pathologist.* He decided very properly that the clinical pathologist in order to be a good hematologist must not only know the individual blood cells but also be skilled in the interpretation and correlation of the clinical laboratory values in the light of the clinical history and physical findings. Riser stated that the clinical pathologist is often regarded as a highly specialized magician imbued with the power of visualizing the complete blood picture upon study of a single blood spread, without previously having any knowledge of the clinical history or physical findings of the patient. The diagnosis can be arrived at only after indicated laboratory studies are completed and careful integration and correlation of all available information have been carried out. Sometimes it is necessary to study the patient over a period of time before the correct diagnosis is evident. The practice of sending blood films to the clinical pathologist with request for hematologic diagnosis is to be decried. It is often necessary for us to write for all the pertinent data before venturing on any conclusions. It is erroneous to use bone marrow studies alone and quite separate from all the other facts of the case as a means of diagnosis. Emphasis is therefore to be laid on the fact that (1) a complete study of the blood must be made from a laboratory standpoint and (2) these data must be correlated very carefully by either the clinician or the clinical hematologist in order to arrive at a probable diagnosis. Without minimizing in any way the importance of the following pages devoted to hematology from a laboratory standpoint, one must bear in mind that a technician cannot possibly be a hematologist and that a clinical pathologist cannot be a hematologist unless he is an accomplished clinician.

Chapter 59

General considerations

JOHN D. BAUER

ORIGIN OF BLOOD CELLS

There still is considerable discussion as to the origin and development of the various blood cells. Certain points are still in controversy as to the monophyletic or polyphyletic development of the principal leukocytes. For an extensive review and bibliography of this subject, we recommend Downey's *Handbook of hematology.*[2] In this handbook the various theories of the origin of blood cells have been exceedingly well portrayed in the section by William Bloom.

Blood formation in embryo and fetus

It is generally agreed that the most primitive blood formation in the early embryo begins with the layer known as **embryonal mesenchyme.** The first cells to be formed in this early period of blood formation are red cells often referred to as **primitive erythroblasts.** In this very early stage or mesoblastic period it is very difficult to find cells that correspond to the white cells of the adult. It is also important to remember that at this stage blood formation takes place within **blood islands** in the yolk sac of the embryo. These early cells are large and have large hyperchromatic nuclei.

At about the second month of fetal development, there begin to appear cells that correspond to the white blood cells in the adult, and, in addition, megakaryocytes can be demonstrated in the liver. At this stage the liver becomes a blood-forming organ and presents red blood cell formation as well as white blood cell formation. These cells originate from the sinusoidal reticulum cells. Associated with this activity of the liver is a modest activity of the spleen, which at first is most concerned with erythropoiesis but later is concerned with the formation of lymphocytes. At about the fifth month of fetal development, the final phase of hematopoiesis is instituted in the fetus. At this point the bone marrow begins to inaugurate its ultimate growth as one of the greatest blood-forming tissues in the human body. Simultaneous with this development of the bone marrow, the lymphatic system and lymph nodes begin the development of lymphocytes (Fig. 333).

In the fetus, the early red cell development or **primitive** development shows cells somewhat similar to the abnormal red cells seen in pernicious anemia. An interesting point about this similarity is that this primitive erythropoiesis gives way to definitive erythropoiesis (the adult type of normal erythropoiesis) at the same time that the fetal liver begins to develop. It is rather interesting that with the administration of liver therapy to patients with pernicious anemia (primitive erythropoiesis), the erythropoiesis reverts to the definitive type.

Blood plasma. Blood plasma can be detected at about the fourth month of intrauterine life. Embryonal plasma contains a higher percentage of globulin than of albumin. Although the mesenchymal origin of the formed blood elements is generally accepted, the origin of the plasma is not well established. Shields[3] recently summarized the existing evidence for the origin of plasma. Cells of mesenchymal origin, by releasing portions of their protoplasm (hyaline bodies) and by disintegrating, produce a sol called the blood plasma. Thus the mesenchyme gives rise to both cellular and lipid elements of the blood.

Leukocytes. Regarding the development of leukocytes, there is still considerable disagreement concerning each type of white blood cell, and as a result, there have appeared several schools of theory concerning the development of leukocytes. Fig. 330 is a chart representation of the various theories of blood formation. These theories, briefly, are divided into 2 major groups, the **monophyletic** and the **polyphyletic** schools.

The monophyletic theory insists that all blood cells, regardless of their character, are derived from a single primitive blood cell that perhaps corresponds to the primitive mesenchyme of the embryo. In the normal adult human being it is difficult, first of all, to find cells that are identical with primitive mesenchymal cells, and it is even more difficult to identify steplike development of such primitive cells through the various forms of leukocytes and erythrocytes. Another strong position that the monophyletists take is that in the adult there are cells that are totipotential, that is, they have the power of developing in any direction. Although this is the complete monophyletic theory, there are other men (Downey, Pappenheim, and Ferrata) who believe in the monophyletic theory but who do not agree that certain cells are totipotential. These men have been labeled "neounitarians" because they believe that, once certain cells have become functionally differentiated, they cannot revert to other cell types.

The polyphyleticists can also be divided into several subgroups, such as **dualists** (2 series of development of white blood cells), **trialists** (3 main stems of development), etc. Ehrlich and Naegeli represent proponents of the dualistic school and Schilling and others, the trialistic.

Suffice it to say at this point that the almost religious fervor in the crusades for various theories of blood formation is now much more a matter of the historic past than of interest to hematologists.

The various theories as to the origin of blood cells have been exceedingly well portrayed in a monograph by Bloom.[4] We are indebted to the author of this monograph and to the publisher for permission to quote from this splendid review of this subject and to quote it freely. Bloom states that the so-called trialism theory of Schilling is about as follows: There proceeds from the mesenchyme cells myeloblastic, lymphoblastic, and histiocytic cells, the last of which become monocytes.

Of Cunningham, Sabin, and Doan's theory, Bloom states: "They derive lymphocytes, granular leukocytes, and monocytes from fixed reticular cells by way of a small round precursor which they call a primitive blood cell.

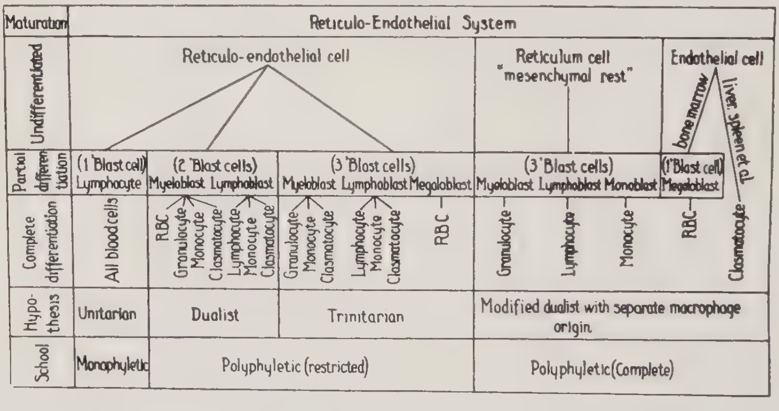

Maturation
Reticulo-Endothelial System
Undifferentiated
Reticulo-endothelial cell
Reticulum cell "mesenchymal rest"
Endothelial cell
bone marrow
liver, spleen et al.
Partial differentiation
(1 Blast cell) Lymphocyte
(2 Blast cells) Myeloblast Lymphoblast
(3 Blast cells) Myeloblast Lymphoblast Megaloblast
(3 Blast cells) Myeloblast Lymphoblast Monoblast
(1 Blast cell) Megaloblast
Complete differentiation
All blood cells
RBC
Granulocyte Monocyte Clasmatocyte
Lymphocyte Monocyte Clasmatocyte
Granulocyte Monocyte Clasmatocyte
Lymphocyte Monocyte Clasmatocyte
RBC
Granulocyte
Lymphocyte
Monocyte
RBC
Clasmatocyte
Hypothesis
Unitarian
Dualist
Trinitarian
Modified dualist with separate macrophage origin
School
Monophyletic
Polyphyletic (restricted)
Polyphyletic (Complete)

Fig. 330. This chart summarizes the principal points in conflict and in agreement between the various theories advanced to explain the histogenesis of the blood cells. There are 3 levels of maturation common to all; in the first level, primitive undifferentiated mesenchymal cells provide the blast cells of the second level, which in turn, by maturation and division, become the completely differentiated cells of the circulating blood, represented by the third level. The degree of polygenesis is determined by the number of partially differentiated (blast) cells believed to be necessary in order to render accordant the experimental facts and observations as interpreted by the various investigators who comprise the schools of thought shown in the lower portion of the chart. The principal difference, as indicated, is in the degree of prepotency ascribed to the blast cells, the unitarians feeling that a single common ancestor (the lymphocyte) may differentiate into any of the mature types of cells. Attributing lesser degrees of potentialities to this cell results in increasing degrees of polyphyletism, which in the extreme construction requires a separate blast cell for each of the circulating elements, and a separation of the reticuloendothelial system of cells into its component units, each with different potentialities.

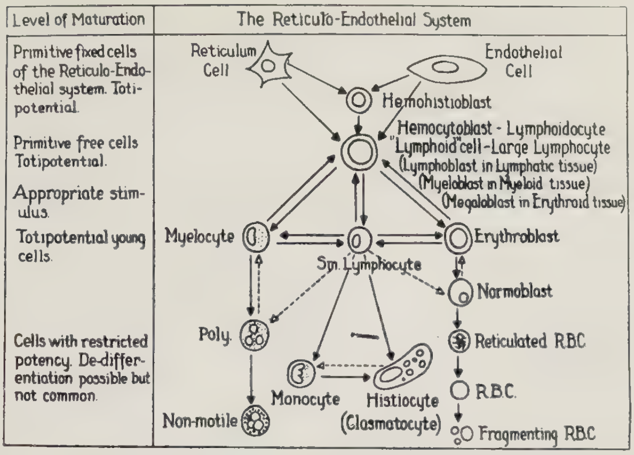

Level of Maturation
The Reticulo-Endothelial System
Primitive fixed cells of the Reticulo-Endothelial system. Totipotential.
Primitive free cells Totipotential.
Appropriate stimulus.
Totipotential young cells.
Cells with restricted potency. De-differentiation possible but not common.
Reticulum Cell
Endothelial Cell
Hemohistioblast
Hemocytoblast - Lymphoidocyte
"Lymphoid" cell - Large Lymphocyte
(Lymphoblast in Lymphatic tissue)
(Myeloblast in Myeloid tissue)
(Megaloblast in Erythroid tissue)
Myelocyte
Sm. Lymphocyte
Erythroblast
Normoblast
Poly.
Reticulated RBC
Monocyte
Histiocyte (Clasmatocyte)
RBC.
Non-motile
Fragmenting RBC

Fig. 331. This chart condenses, by diagram, the monophyletic interpretation of the literature that is concerned with the relationships between the cells of the blood and those of the tissues. A common undifferentiated totipotential cell is postulated and identified with the hemocytoblast of Ferrata, the lymphoidocyte of Pappenheim, and the blood lymphocyte, lymphoblast, myeloblast, megaloblast, and monoblast of the polyphyletic adherents. In theory the small lymphocyte of the blood is believed simply to be a temporary resting phase of the larger lymphoid cell and may differentiate into the other cells, as shown, either directly or after reverting back to the large tissue cell of the Ferrata type. The small or large lymphocyte may also develop into the more mature blood cells directly without going through the maturation phases of these cells, and, finally, after having so differentiated, it may dedifferentiate back to the primitive totipotential level. The reticulum and endothelial cells of the reticuloendothelial system are believed to be potentially identical, differing only in shape. They give rise to the lymphocytes of the blood, either directly or through a primitive tissue cell similar to the hemohistioblast of Ferrata. Opinion varies between different supporters of the monophyletic school as to the importance of the transition from monocyte and clasmatocyte back to lymphocyte, as well as other minor points intentionally omitted on this chart, in order to emphasize the important concepts involved in this theory of blood formation.

The formation of granulocytes takes place in the bone marrow, that of monocytes in the spleen, and of lymphocytes in spleen and lymph nodes. These authors also describe the development of the red blood cells and macrophages (clasmatocytes) from endothelium."*

The dualistic theories of Ehrlich, Schridde, and Naegeli derive the blood cells from 2 sources, myelocytic and lymphocytic.

The extreme unitarians, according to Bloom, are Dominici, A. Wolff, Ciaccio, Maximow, Dantschakoff, Weidenreich, Latta, Jordan, Jolly, Jordan and Spiedel, Bloom, Kingsbury, and Maximow and Bloom. They believe that the 3 stem cells of the connective, lymphatic, and myeloid tissues of the adult and of the various sites of blood formation in the embryo are representatives of a single cell type. Maximow adopted the term **hemocytoblast** for the free stem cell.

There are so-called neounitarian theories, of which Pappenheim, Ferrata, and Downey are the leading exponents. They uphold the unitarian theory of blood cell formation and admit to a greater or lesser degree the morphologic identity of the free stem cells in the myeloid and lymphatic tissues. They believe that the cells in their respective situations are functionally differentiated.

It may be well to summarize in tabulated form the various and differing conceptions of the origin of the white blood cells. Wiseman[5] (Fig. 330) summarizes the principal points that are in conflict and in agreement between the various theories that have been advanced to explain the histogenesis of the blood cells. In another chart (Fig. 331) Wiseman shows the monophyletic interpretation of the literature that is concerned with the relationships between the cells of the blood and those of the tissues.

A recapitulation of the preceding shows blood cells, according to the unitarian group, coming from undifferentiated mesenchymal cells; according to the dualists, the sources are myelocytic and lymphocytic, and some origin from the histiocytes of the tissues. The trialism school, which is headed by Schilling

---

*From Bloom, W.: Lymphocytes and monocytes: Theories of hematopoiesis. In Downey, H. (editor): Handbook of hematology, New York, 1938, Paul B. Hoeber, Inc., Medical Book Department of Harper & Brothers, vol. 1, pp. 419-435.

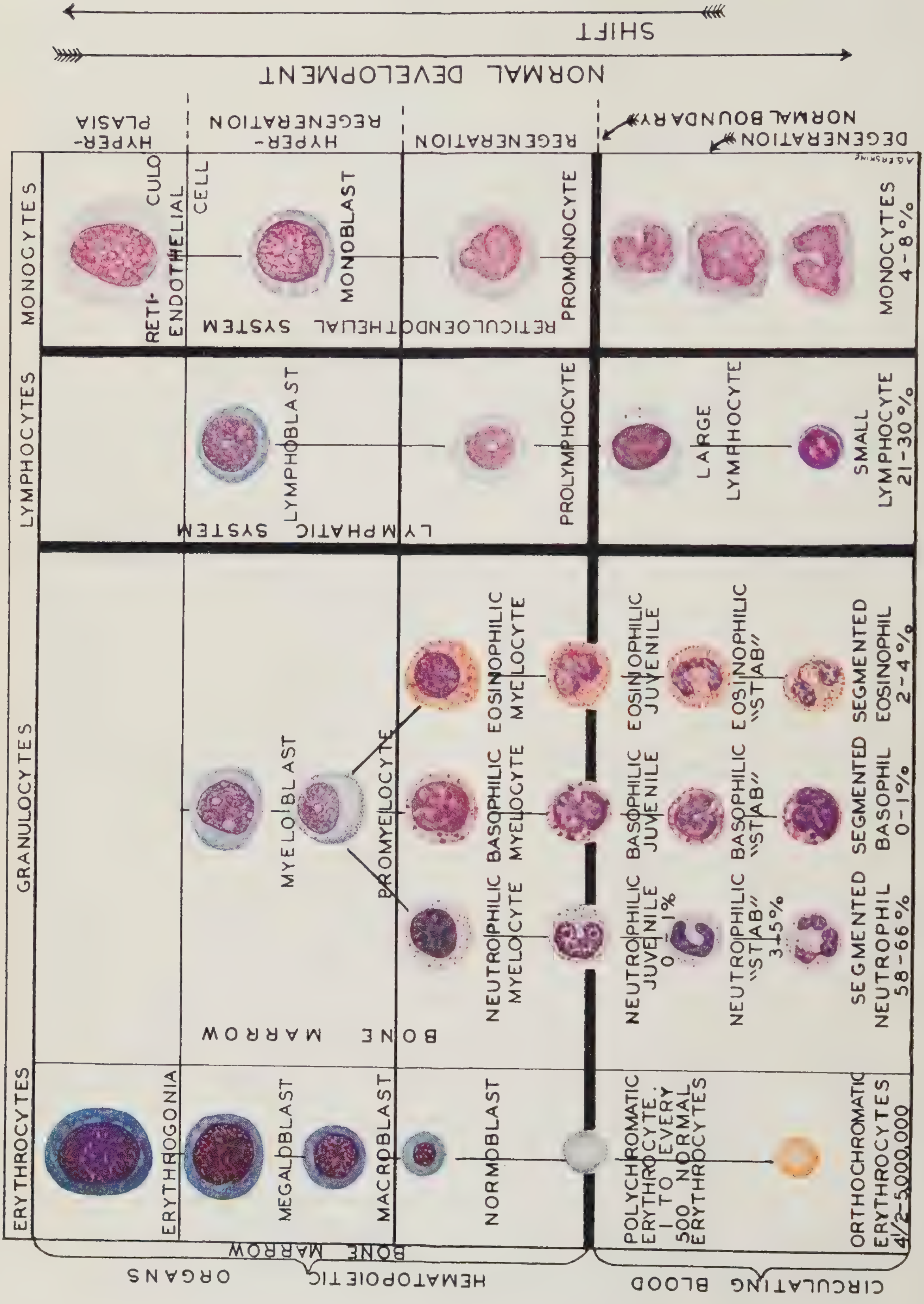

SHIFT
NORMAL DEVELOPMENT
HYPER-PLASIA
HYPER-REGENERATION
REGENERATION
NORMAL BOUNDARY
DEGENERATION
MONOCYTES
RETI-CULO ENDOTHELIAL CELL
MONOBLAST
PROMONOCYTE
MONOCYTES 4-8%
RETICULOENDOTHELIAL SYSTEM
LYMPHOCYTES
LYMPHOBLAST
PROLYMPHOCYTE
LARGE LYMPHOCYTE
SMALL LYMPHOCYTE 21-30%
LYMPHATIC SYSTEM
GRANULOCYTES
MYELOBLAST
PROMYELOCYTE
NEUTROPHILIC MYELOCYTE
BASOPHILIC MYELOCYTE
EOSINOPHILIC MYELOCYTE
NEUTROPHILIC JUVENILE 0-1%
BASOPHILIC JUVENILE
EOSINOPHILIC JUVENILE
NEUTROPHILIC "STAB" 3-5%
BASOPHILIC "STAB"
EOSINOPHILIC "STAB"
SEGMENTED NEUTROPHIL 58-66%
SEGMENTED BASOPHIL 0-1%
SEGMENTED EOSINOPHIL 2-4%
BONE MARROW
ERYTHROCYTES
ERYTHROGONIA
MEGALOBLAST
MACROBLAST
NORMOBLAST
POLYCHROMATIC ERYTHROCYTE, 1 TO EVERY 500 NORMAL ERYTHROCYTES
ORTHOCHROMATIC ERYTHROCYTES 4½-5,000,000
ORGANS
HEMATOPOIETIC BONE MARROW
CIRCULATING BLOOD
AGERSKINE

Fig. 332. Diagram of normal development of blood cells (Schilling) explaining pathologic shifts.

and his followers, believes in three points of development of white cells. Fig. 332 illustrates the Schilling trialism theory.

Whatever the theories, the biologic fact remains that the first white cells appear at about 2 mo. They are myeloblasts. At 3 mo., granulocytes (mainly eosinophils), lymphocytes, and megakaryocytes can be detected in the fetal blood. In the fifth month, monocytes make their appearance. There is good evidence that all of these cells are produced by the reticuloendothelial system.

NOMENCLATURE OF HEMATOLOGY

There is considerable confusion in hematologic literature due to terminology. Osgood suggested a nomenclature for the erythrocyte and granulocyte series because of the disagreement in definitions and the inappropriateness of the terms in current use. He called attention, for instance, to the term **normoblast,** which is a combination of a Latin and a Greek root which should mean a normal stem cell since the termination blast is ordinarily employed only for the most immature cells of a series. The cell, however, is neither a stem cell nor a normal cell of the blood. He further maintains very properly that the derivation for the erythrocyte series is from the Greek word meaning **nucleated.** The old term **myeloid series,** meaning marrow-like cells, is a misnomer because these are cells forming an integral part of the marrow. This does not differentiate them from cells of the monocyte, plasmacyte, or erythrocyte series which are also found normally in the marrow. Osgood believes, as we do, that the term **granulocyte series** is more logical for all cells of this group.

Because of the confusing terminology that has developed in the field of hematology, a special committee of outstanding hematologists from various parts of the United States met in Chicago in October, 1947, for the purpose of attempting to clarify the nomenclature of cells and diseases of the blood and blood-forming organs. The major recommendations are listed in Tables 221-223. "**It is not the intention of the Committee to imply from its recommendation of terms to be used that the origin of all these cells has been settled.**"

It should be mentioned here that not all of the members of the Committee were in entire accord as to the final nomenclature to be used, but they felt that some modicum of agreement would help eliminate some of the confusion in the field of hematology. Despite the efforts of this group to unify the nomenclature, there are certain terms of historic use that will be difficult to eliminate from the writing and speaking of hematologists the world over.

Weicker,[6] on the basis of his own and others' investigations, suggested a karyometric method of classification of cells based on mathematical nuclear volume studies and on the fact that, after nuclear division, the volumes of the daughter nuclei are always in a

Table 221. *Recommended terms and terms to be avoided when referring to specific cells of the erythrocytic series**

Term to be used	*Terms to be avoided*
Rubriblast	Erythroblast, megaloblast, pronormoblast, promegaloblast, normoblast, hemocytoblast, stem cell, myeloblast, lymphoidocyte, karyoblast
Prorubricyte	Erythroblast, megaloblast, pronormoblast, normoblast, macronormoblast, macroblast, prokaryocyte
Rubricyte	Normoblast, pronormoblast, macronormoblast, erythroblast, polychromatophilic normoblast, karyocyte
Metarubricyte	Normoblast, erythroblast, metakaryocyte
Reticulocyte†	
Erythrocyte	Red blood cell, erythroplastid, normocyte, akaryocyte

*From Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-Forming Organs, Am. J. Clin. Path. **19**:56-59, 1949.

†It is recommended that the reticulocyte stage be considered a subdivision of the erythrocyte stage.

Table 222. *Recommended terms and terms to be avoided when referring to cells of a particular series or to a disease affecting any cell of that series**

Term to be used	*Terms to be avoided*
Lymphocytic	Lymphoid, lymphatic, lymphogenous, lymphocyte, mononuclear
Granulocytic	Myeloid, myelogenous, myelocyte, myelocytic, granulocyte, leukocyte, leukocytic, leucocyte, leucocytic
Monocytic	Monocytoid, monocytogenous, mononuclear, monocyte
Plasmacytic	Plasma cellular, plasmacytogenous, myeloma cell, plasmacyte
Erythrocytic	Erythroid, erythrocytoid, erythron, erythrocytogenous, erythrocyte
Thrombocytic	Megakaryocytic, platelet, thrombocyte

*From Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-Forming Organs, Am. J. Clin. Path. **18**:443-450, 1948.

Table 223. *Recommended terms and terms to be avoided when referring to specific cells of the blood and blood-forming organs**

Name of series	*Term to be used*	*Terms to be avoided*
Lymphocytic	Lymphoblast	Myeloblast, hemocytoblast, lymphoidocyte, stem cell, lymphocyte
	Prolymphocyte	Large lymphocyte, pathologic large lymphocyte, atypical leukocytoid lymphocyte, monocyte, immature lymphocyte
	Lymphocyte	Small, medium or large lymphocyte, normal lymphocyte, small, medium or large mononuclear
Monocytic	Monoblast	Myeloblast, hemocytoblast, lymphoidocyte, lymphocyte, stem cell, immature monocyte
	Promonocyte	Premonocyte, hemohistioblast, immature monocyte, Ferrata cell
	Monocyte	Large mononuclear, transitional, clasmatocyte, endothelial leukocyte, histiocyte, resting wandering cell
Granulocytic	Myeloblast	Granuloblast, hemocytoblast, lymphoidocyte, lymphocyte, stem cell
	Progranulocyte	Promyelocyte II, leukoblast, myeloblast, premyelocyte, promyelocyte, progranulocyte A
	Myelocyte	Granulocyte, myelocyte B, nonfilament, class I
	Metamyelocyte	Metagranulocyte, juvenile, myelocyte C, nonfilament, class I
	Band cell	Staff cell, stab cell, nonfilament, class I, rod nuclear, polymorphonuclear, stabkernige, rhabdocyte, nonsegmented
	Segmented	Polymorphonuclear, filamented, class II, III, IV, or V, lobocyte
Plasmacytic	Plasmablast	Myeloblast, hemocytoblast, lymphoidocyte, lymphocyte, stem cell, lymphoblastic plasma cell, myeloma cell
	Proplasmacyte	Türck cell, Türck irritation form, lymphoblastic or myeloblastic plasma cell, myeloma cell
	Plasmacyte	Plasma cell, Unna plasma cell, Marschalko plasma cell, plasmacytoid lymphocyte, myeloma cell
Thrombocytic	Megakaryoblast	Megalokaryoblast
	Promegakaryocyte	Premegalokaryocyte
	Megakaryocyte	Megalokaryocyte
	Thrombocyte	Platelet, thromboplastid
	Disintegrated cell	Senile cell, smudge, basket cell, smear cell, degenerated cell

*From Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-Forming Organs, Am. J. Clin. Path. **18**:443-450, 1948.

constant proportion to each other and to the mother nucleus.

In the erythroid series, this classification would be as follows:

If the letter K represents the nuclear volume:

K_2 represents an early erythroblast with nuclear diameter of 13μ

K_1 represents a late erythroblast with a nuclear diameter of 10.3μ

$K_{1/2}$ is assigned to a basophilic normoblast with a nuclear diameter of 8.2μ

$K_{1/4}$ corresponds to a polychromatic normoblast with nuclear diameter of 6.5μ

$K_{1/8}$ signifies an oxyphilic normoblast with a nuclear diameter of 5.2μ

In the granulocytic series, the classification would be as follows:

K_1 is myeloblast with nuclear diameter of 15μ

K_2 is promyelocyte

$K_{1\text{-}1/2}$ is myelocyte

$K_{1/2\text{-}1/3}$ is granulocyte

Glossary of common hematologic terms

hematopoietic organs The blood-forming organs.

erythropoiesis The manufacture of red blood cells.

erythrocytes Red blood corpuscles.

normocytosis A condition characterized by the presence of normal-sized erythrocytes *(normocytes).*

microcytosis A condition characterized by the presence of small erythrocytes *(microcytes).*

macrocytosis A condition characterized by the presence of large erythrocytes *(macrocytes).*

anisocytosis Abnormal variation in the size of erythrocytes.

poikilocytosis The appearance of abnormally shaped erythrocytes: pear-shaped, oak-leaf, club, or crenated.

crenation The scalloped or notched appearance of the edges of erythrocytes found in pathologic conditions, or caused by undue pressure when making a blood film; also noted when erythrocytes are suspended in hypertonic solutions.

normochromia or *normochromasia* Normal pigment content of erythrocytes.

hypochromia or *hypochromasia* Decreased pigment content of erythrocytes.

hyperchromia or *hyperchromasia* Increased pigment content of erythrocytes.

polychromasia The presence in the fixed blood film of blue-gray-staining erythrocytes.

basophilic punctation or *basophilic stippling* A characteristic of mature erythrocytes which, under certain toxic conditions, show dark staining of punctate bodies in the cytoplasm, demonstrable by routine staining methods.

reticulocyte A slightly immature red cell containing a dark-staining weblike or lacelike structure, which can be demonstrated only by some type of supravital staining.

basophilia of the erythrocytes The appearance in the circulating blood of immature erythrocytes which show bluish gray staining of the cytoplasm demonstrable by routine staining technics.

normoblast A nucleated red blood cell.

megaloblast An abnormal primitive nucleated red blood cell.

hemacytometers Instruments used in counting blood cells.

anemia A symptom complex accompanied by reduction in red blood cells, hemoglobin, and red cell volume per cent of variable degree.

oligocytosis, or *hypoglobulia,* or *erythropenia,* or *oligocythemia* A low red blood cell count.

erythrocytosis, or *polycythemia,* or *hyperglobulia* An elevated red blood cell count.

rouleaux formation of erythrocytes The tendency of erythrocytes to lie with overlapping edges or in roll formation, such as may be seen when a stack of coins is pushed over.

hemoglobin An iron-bearing protein which gives the red color to blood.

hemoglobinometers Instruments used for measuring hemoglobin.

oxyhemoglobin The bright red hemoglobin that is loosely combined with oxygen and present in the arteries.

reduced hemoglobin A combination of hemoglobin and carbon dioxide and present in venous blood.

leukopoiesis The manufacture of white blood cells.

leukocytes White blood cells.

leukocytosis A high white blood cell count.

neutrophilia A high neutrophil count.

lymphocytosis A high lymphocyte count.

monocytosis A high monocyte count.

eosinophilia A high eosinophil count.

hyperleukocytosis Leukocytosis over 25,000.

leukopenia A low white blood cell count.

neutropenia A low neutrophil count.

lymphocytopenia or *lymphopenia* A low lymphocyte count.

monocytopenia or *monopenia* A low monocyte count.

hypoeosinophilia or *eosinopenia* A low eosinophil count.

toxic granules Large irregularly clumped granules seen in the cytoplasm of neutrophils in disease processes such as severe bacterial invasion and chemical intoxication.

hemogram A complete picture of the blood, including numerical and differential counts, hemoglobin estimations, blood platelet counts, reticulocyte counts, special tests, and complete examination of stained erythrocytes.

hemogram of Schilling A linear depiction of the differential count of leukocytes so arranged as to show at a glance a pathologic shift in the differential picture.

nuclear shift An increase or decrease in the number of neutrophilic cells of one type over another.

degenerative leukocytic blood picture One which shows an increase in the number of stab cells over normal (together with other findings) due to a toxic depressing influence on the hematopoietic organs.

regenerative leukocytic blood picture One which shows an increase in the number of juvenile or metamyelocyte cells over normal or an occasional myelocyte, usually with an increased white blood cell count due to stimulation of leukopoiesis.

leukemoid or *liberation* or *flooding leukocytic blood picture* One which shows a marked increase in the number of myelocytes, juvenile, and stab cells over normal cells signifying total loss of defense of the hematopoietic organs; nucleated red blood cells may be seen; must be differentiated from leukemia.

leukemia A fatal disease of unknown origin which primarily involves white blood cells which may or may not be increased in numbers in the blood stream; pathologic forms of leukocytes, often very young, are always present in this disease.

blood platelets (thrombocytes) Small bodies found in the circulating blood which bear an important relationship to blood clotting.

thrombocytosis A high blood platelet count.

thrombopenia or *thrombocytopenia* A low blood platelet count.

hyperplasia An overgrowth of tissue; in reference to blood formation may refer to any or all of the blood elements; most strikingly seen in the leukemias.

hypoplasia Diminished tissue formation.

aplasia Cessation of tissue formation.

blood dust or *hemokoniae* Small refractive bodies in the blood.

chemotaxis Phenomenon shown by certain living cells of moving toward (positive) or away from (negative) certain other cells or substances which exert a chemical influence.

agglomeration Adhering together of cells of the same kind, such as rouleaux formation of erythrocytes, groups of white cells, thrombus formations of blood platelets, heaping of eosinophils, etc.; important in sedimentation tests and in the making of blood spreads.

mitosis Indirect cell division through nuclear change or *karyokinesis;* the typical mode of vision of active somatic cells and germ cells.

HEMATOPOIETIC ORGANS AND THEIR PHYSIOLOGY

Embryonal blood development

Blood cell formation takes place primarily within the body in the embryonal state in the area vasculosa. From this emanate angioblasts. The nomenclature in this stage of embryonal blood development is still in controversy. Minot terms the first intravasal cells **mesamoeboid** cells and believes them to be the parent cells of all blood cells (Pappenheim, Maximow, Dantschakoff, Naegeli, Schridde). These cells are transformed into erythroblasts by the formation of hemoglobin; in the human embryo, this occurs at the 8 mm. stage of development. Sabin noted a very early genetic separation of granulocytic, monocytic, and lymphocytic centers. She believed that granulocytes came into existence from the direct transformation of angioblasts, and that lymphocytes originated from body

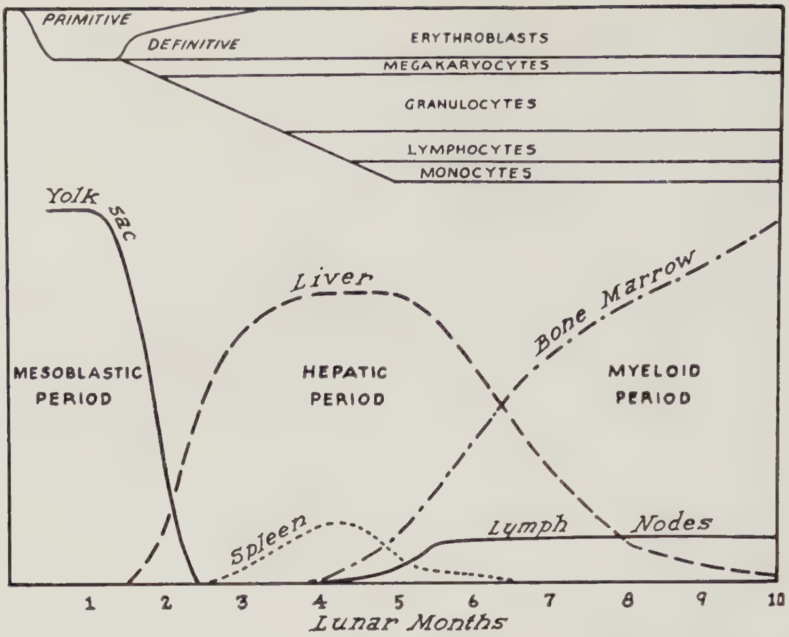
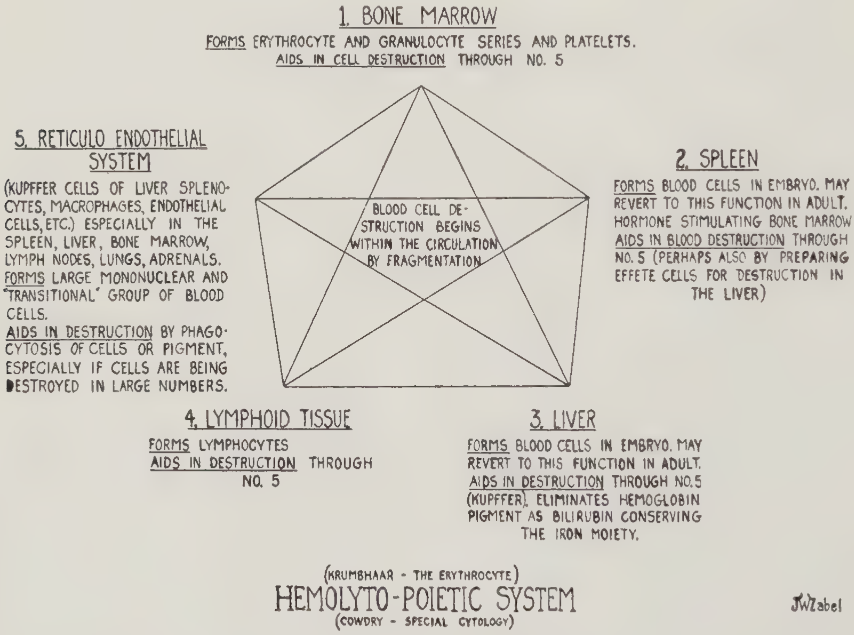


Fig. 333. Stages of hemopoiesis in embryo and fetus, indicating comparative participation of chief centers of hemopoiesis and approximate times at which different types of cells make their appearance. (From Wintrobe, M. M.: Clinical hematology, Philadelphia, 1952, Lea & Febiger.)


1. BONE MARROW

FORMS ERYTHROCYTE AND GRANULOCYTE SERIES AND PLATELETS.
AIDS IN CELL DESTRUCTION THROUGH NO. 5

5. RETICULO ENDOTHELIAL SYSTEM

(KUPFFER CELLS OF LIVER SPLENOCYTES, MACROPHAGES, ENDOTHELIAL CELLS, ETC.) ESPECIALLY IN THE SPLEEN, LIVER, BONE MARROW, LYMPH NODES, LUNGS, ADRENALS.
FORMS LARGE MONONUCLEAR AND "TRANSITIONAL" GROUP OF BLOOD CELLS.
AIDS IN DESTRUCTION BY PHAGOCYTOSIS OF CELLS OR PIGMENT, ESPECIALLY IF CELLS ARE BEING DESTROYED IN LARGE NUMBERS.

BLOOD CELL DESTRUCTION BEGINS WITHIN THE CIRCULATION BY FRAGMENTATION

2. SPLEEN

FORMS BLOOD CELLS IN EMBRYO. MAY REVERT TO THIS FUNCTION IN ADULT. HORMONE STIMULATING BONE MARROW
AIDS IN BLOOD DESTRUCTION THROUGH NO. 5 (PERHAPS ALSO BY PREPARING EFFETE CELLS FOR DESTRUCTION IN THE LIVER)

4. LYMPHOID TISSUE

FORMS LYMPHOCYTES
AIDS IN DESTRUCTION THROUGH NO. 5

3. LIVER

FORMS BLOOD CELLS IN EMBRYO. MAY REVERT TO THIS FUNCTION IN ADULT.
AIDS IN DESTRUCTION THROUGH NO. 5 (KUPFFER). ELIMINATES HEMOGLOBIN PIGMENT AS BILIRUBIN CONSERVING THE IRON MOIETY.

(KRUMBHAAR - THE ERYTHROCYTE)
HEMOLYTO-POIETIC SYSTEM
(COWDRY - SPECIAL CYTOLOGY)

Fig. 334. Blood cell formation and destruction according to Maximow.

cells at a considerably later period than the other cells just described.

The first localization of hematopoiesis may be observed in the liver of the 12 mm. embryo, which is in the third month of embryonal life. At this time there begins the development of the outstanding blood organ, namely, the bone marow. While it is true that the differentiation of cells occurs in early embryonal stages, physiologic regeneration of injured or lost blood cells later in life suggests the possibility of later new differentiation of various cell types from certain elements that remain indifferent and latent, that is, mesenchymatous or angioblastic cells. Blood cells develop outside the sinusoids from mesenchymal cells (extravascularly), as well as within the sinusoids from endothelial cells (intravascularly).

Bone marrow formation begins in the third embryonal month and soon changes into myelocytic elements. Transformation of connective tissue cells into lymphoid wandering cells soon ceases, and differentiated tissue—erythroblasts, myeloblasts, and giant cells—appears. Erythroblastic and megaloblastic cells soon are seen. Granulocytic elements are scarce at first and are isolated or in small groups. The differentiation of basophilic and eosinophilic cells in the bone marrow is a very early phenomenon. Embryonal bone marrow is very rich in cells and remains so in the first years of life, gradually changing as infancy begins. It is to be noted that the bone marrow of the long bones in embryos and children is more hematopoietic than that of adults. Attention will later be called to the fact that bone marrow may be changed into fat and may change back again into red marrow. Red marrow is found in all bones of newborn infants, decreasing in the child up to the end of the seventh year. Gradual concentration of the marrow into the smaller bones occurs, and red marrow is seen in adult life in the proximal epiphyses of the long hollow bones, the skull bones, the ribs, the sternum, the scapulae, the clavicles, the vertebrae, and the flat pelvic bones.

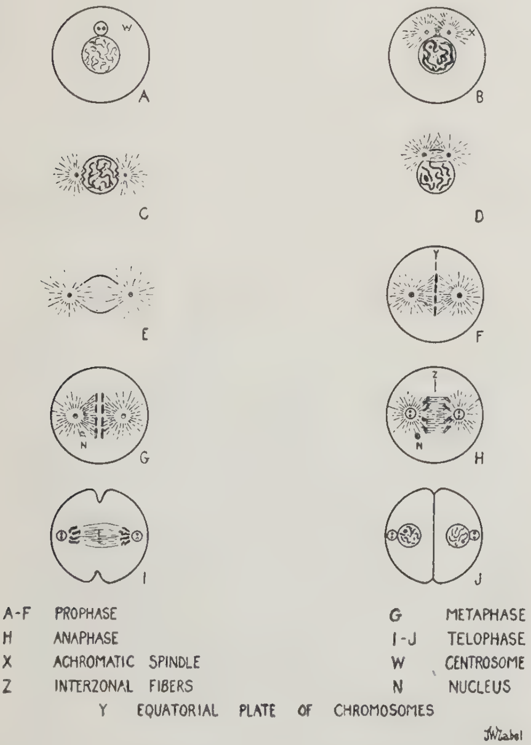


Fig. 335. Successive stages of mitosis. (After Wilson.)

In the human being after birth, hematopoiesis is concentrated in the bone marrow and the lymph nodes. Normal bone marrow is the primary source of erythrocytes, cells of the myelocytic series, and platelets. Lymph nodes are the major source of lymphocytes, with the spleen contributing to the production of these cells.

General facts on cellular development

All cells arise from pre-existing cells by division. This fact was first brought out in the celebrated dictum of Rudolf Virchow: "*omnis cellula e cellula.*" Cells divide in 2 ways—by amitosis and by mitosis. Amitosis is direct division of cells by the simple fission of nucleus and cytoplasm. It is not preceded by structural metamorphosis of the nucleus or cytoplasm. In mitosis complicated changes take place in the nucleus. Since these changes give rise to threadlike structures developed out of the nuclear chromatin, the process is termed mitosis from the word **thread,** in contrast to amitosis or **no thread.** The distinctive feature of mitosis is the production of a characteristic number of chromosome bodies, their accurate lengthwise halving, and the equal distribution of these halves to the daughter cells.

Mitosis or **karyokinesis** was first discovered by Schleicher and was studied first by Flemming. It is the most important method of cell division. There are different types of indirect generation. The phases of the process, beginning with the state of rest, are classified as progressive and regressive. The progressive changes of structure occur between rest and the phase of metakinesis or mother star. The regressive retrocede from the height of metakinesis to the resting stage of the daughter cells. The order and nomenclature of the phases are as follows.

First, the **progressive phases** occur. These are divided into the **prophase** and the **metaphase.** In the prophase stage there are (1) the phase of rest, (2) the phase of gross segmentation, and (3) the phase of the ovum. In the metaphase stage there are the phase of transverse segmentation and the phase of the mother star or metakinesis. In the **prophase** the centrioles separate from each other with the formation of attraction spheres around each of them while the chromatic masses change into **chromosomes,** the number of which is constant in a given species. In man, there are 46 chromosomes (22 pairs of autosomes, nonsex chromosomes, and 2 sex chromosomes, XX in the female and XY in the male). In the **metaphase** each chromosome splits into 2 equal parts.

There are 2 states in the regressive phases, the **anaphase** and the **telophase.** In the **anaphase** the halves of a particular chromosome separate and each half approaches 1 of the 2 centrioles which have migrated in the meantime to opposite poles of the cell. In the **telophase** the formation of the 2 daughter nuclei and the division of the cytoplasm into masses surrounding each daughter nucleus takes place.

In the **prophase** state, the chromatic net of the nucleus becomes richer, and the nucleus increases in size. The idiozome and the centrioles become visible. Reticulation occurs preparatory to the organization of the ovum.

In the **phase of the ovum,** there are seen 2 classes of trabeculae, primary or gross, formed principally from the chromatin, and secondary or fine, constituted by the linin threads. The primary filaments or chromosomes are disposed in hairpinlike forms oriented almost always in the same direction, with the elbows turned to one side. The site of the nucleus at which the angles of the hairpins converge presents a space devoid of filaments, the polar field. The opposite extreme, the antipode or contrapolar field, is filled by the accumulation and intercrossing of the free ends of the chromosomes.

At the end of the **spireme stage** the chromatic filament divides into the number of chromosomes characteristic of the species of the cell. These chromosomes assume the form of hairpins.

During the **metaphase** or in metakinesis the chromatic threads or hairpins are arranged in the form of a star. Another characteristic of this phase is the enlargement and dislocation of the achromatic spindle by the division of the chromosomes. The chromosomes dispose themselves around the widest region of the spindle in the form of a star at the perpendicular or equatorial plane of which occurs the segmentation of the nucleus and protoplasm.

In the **anaphase** the chromosome hairpins are duplicated and begin to separate from the elbows toward the extremities. There is now seen a running of the achromatic handles toward the poles forming a double star or diaster.

In the **telophase** the centrosomes and the spindle disappear while a fine membrane forms around the daughter chromosomes. The nucleus takes the form of a horseshoe. This protoplasmic furrow reaches the equator of the spindle and forms a thin bundle of very fine threads. Shortly after this, the bundle bursts and the intermediary body disappears.

In direct or **amitotic** division the nucleus is thought to constrict and finally to divide into 2 halves, a nucleolus going to each daughter nucleus. There is much discussion as to whether this process actually occurs under normal conditions.

The physiologic basis of nuclear division is the 2-fold increase of nuclear deoxyribonucleic acid. Various methods of division have been described. If the daugther cells are replicas of the mother cell, we speak of **homoplastic division.** If the daughter cells differ from the mother cell, we speak of **heteroplastic division.** Many subdivisions are possible; e.g., one daughter cell may develop to become a cell similar to the mother cell, whereas the other daughter cell may become a different type of cell; both daughter cells may develop into cells which differ from the maternal cells; while, last, the daughter cells may differ not only from the mother cell, but also from each other.

A knowledge of cell division and chromosomes has gained increased importance with the advent of phase microscopy, electron microscopy, and clinical methods of chromosome counting. The first 2 methods allow one to visualize chromosome activity and composition and to investigate the action of cytotoxic drugs, whereas the latter method allows genetic evaluation of disease processes.

Pathology of cell division

Many of the cytotoxic drugs used in the fight against cancer (and therefore against leukemia) attack cells in mitosis or attack the chromosomes. Colchicine and cortisone damage the cell in mitosis, whereas the folic acid antagonist aminopterin injures the chromosomes.

In hematology, microblasts and abnormal giant cells represent instances of abnormal cell division, whereas megaloblastic anemias, aplastic anemias, aplastic crises of hemolytic anemias, and agranulocytosis represent instances of arrest of cell division at various levels.

HUMAN CHROMOSOMES[7-14]

Chromosomes can be divided into the **diploid autosomal chromosomes,** which have no relation to sex, and the **haploid sex chromosomes,** which, as the name implies, determine the sex of the individual.

The chromosomes consist of deoxyribonucleic acid nucleoprotein which carries the genes, the hereditary units. With the exception of the germ cells, all somatic cell nuclei of a given species contain a constant amount of deoxyribonucleic acid. All chromosomes are diploid with the exception of the chromosomes of the mature germ cell, which are haploid. The mitotic division as described applies to all chromosomes of the female organism and to 22 pairs of the 23 pairs of the male chromosomes.

Sex chromosomes

In man there is present an X chromosome and a Y chromosome. The Y chromosome is responsible for the masculine sex characteristics. The mature male germ cell contains 22 single autosomes and either an X chromosome or a Y chromosome. If a female germ cell, which carries a single X sex chromosome, receives a Y chromosome from the male germ cell, the offspring will be of the male sex. If the female germ cell receives an X chromosome, the child will be female.

The female cell is characterized by a pair of like XX chromosomes, whereas the male cell has a pair of unlike chromosomes XY. The recognition of the sex chromatin in the cell allows the investigation of cases in which the chromosomal sex is suspected to differ from the anatomic sex.

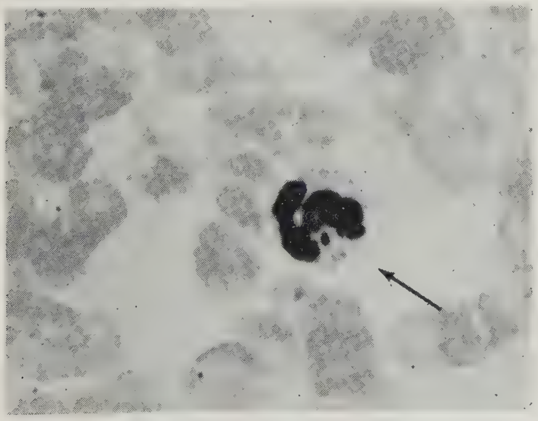


Fig. 336. Drumstick in polymorphonuclear leukocyte of female. (From Bauer, J. D., Toro, G., and Ackermann, P. G.: Bray's clinical laboratory methods, St. Louis, 1962, The C. V. Mosby Co.)

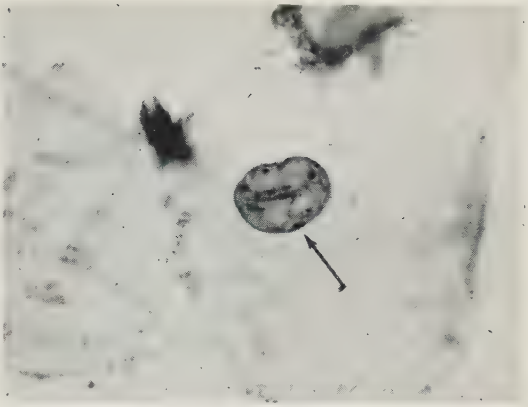


Fig. 337. Buccal epithelium revealing sex chromatin body. (From Bauer, J. D., Toro, G., and Ackermann, P. G.: Bray's clinical laboratory methods, St. Louis, 1962, The C. V. Mosby Co.)

In the individual cell, the **Barr body** or sex chromatin body probably represents the second X chromosome and is therefore not seen in the cells of the male but is detected only in the cells of the female. In the epithelial cell it appears as a mass of biconvex chromatin measuring about 0.7-1.2μ in diameter, situated within the nucleus so closely applied to the inside of the nuclear membrane that it cannot be separated from it even under oil immersion.

Methods of nuclear sexing

Several methods of nuclear sexing can be employed: (1) skin biopsy, (2) buccal smear, and (3) peripheral blood film.

Skin biopsy and buccal smear methods are outside the field of hematology but references are given because the peripheral blood film method should always be complemented by one or the other additional method.

Peripheral blood film technic

In at least 6 out of every 500 neutrophilic polymorphonuclear leukocytes in the female there is a small drumsticklike projection attached to the nucleus. This projection, made of nuclear chromatin, has a small, slightly ovoid head measuring 1.5μ in diameter and is attached to the nucleus by means of a delicate threadlike structure. It can also be seen in the eosinophils and basophils. If strict criteria are employed, the frequency of drumsticks in polymorphonuclear cells and of sex chromatin in buccal cells is comparable. Strict criteria are necessary since in the male polymorphonuclear cell variously sized and shaped nuclear projections can be seen. Some resemble drumsticks; others are triangular and pointed. It is conceivable that the drumstick represents a genetically determined sex characteristic rather than the actual sex chromosome. The technic employs a regular thin blood film stained with Wright or Giemsa stain.

Sex chromosomal defects include gonadal dysgenesis (Turner's syndrome and Klinefelter's syndrome), true hermaphroditism, and superfemale.

Turner's syndrome is due to ovarian dysgenesis. Anatomically these patients are females, but the cellular chromosome pattern is negative (XO). At puberty, they fail to develop female sexual characteristics and often show webbing of the neck. The ovaries consist mainly of stroma.

Klinefelter's syndrome is due to testicular dysgenesis, and though the anatomic build is male, the nuclear chromatin pattern is female (XXY). At puberty, these boys fail to develop. The testes consist mainly of hyalinized connective tissue and Sertoli cells.

Both syndromes are causes of sterility.

In true hermaphroditism, the gonad is an ovotestis; therefore the chromosomal sex may be either XX, XY, or mosaic (both).

The superfemale pattern XXX is probably due to nondysjunction of the sex chromosome during the formation of the parental cells. Nondysjunction can also explain Klinefelter's and Turner's syndromes as well as some lethal combination such as YO and XYY.

Autosomal chromosomes

Recently introduced methods of cell culture which allow the analysis of human chromosomes at metaphase have shed new light on many diseases and have opened an entirely new field of investigation. These investigations touch upon hematologic problems since abnormal karyotypes have been described in such conditions as hemophilia, leukemia, and Gaucher's disease and following irradiation. The chromosomes can be visualized only during nuclear division in metaphase. Each chromosome is characterized by its over-all length, by the length of its arms, and by the position of the centromere. Each chromosome has an identical mate in the nucleus irrespective of how they are arranged. They can be photographed, cut out, arranged in identical pairs, and numbered according to the Denver system (human karyotype). As already discussed, in the male the 2 sex chromosomes are not identical (X, Y), whereas in the female they are (XX). Various genetic mechanisms may be responsible for chromosomal abnormalities. Nondysjunction, as has been described in the *Drosophila,* implies that in spermatogenesis, oogenesis, or mitosis a chromosome pair fails to separate and enters the nucleus of the daughter cell together so that one daughter cell receives an extra chromosome at the expense of the other.

Mosaicism (chimera) means that the individual consists of cells of 2 or 3 chromosomal types. Another mechanism is simple loss of a chromosome.

Chromosomal abnormalities have been de-

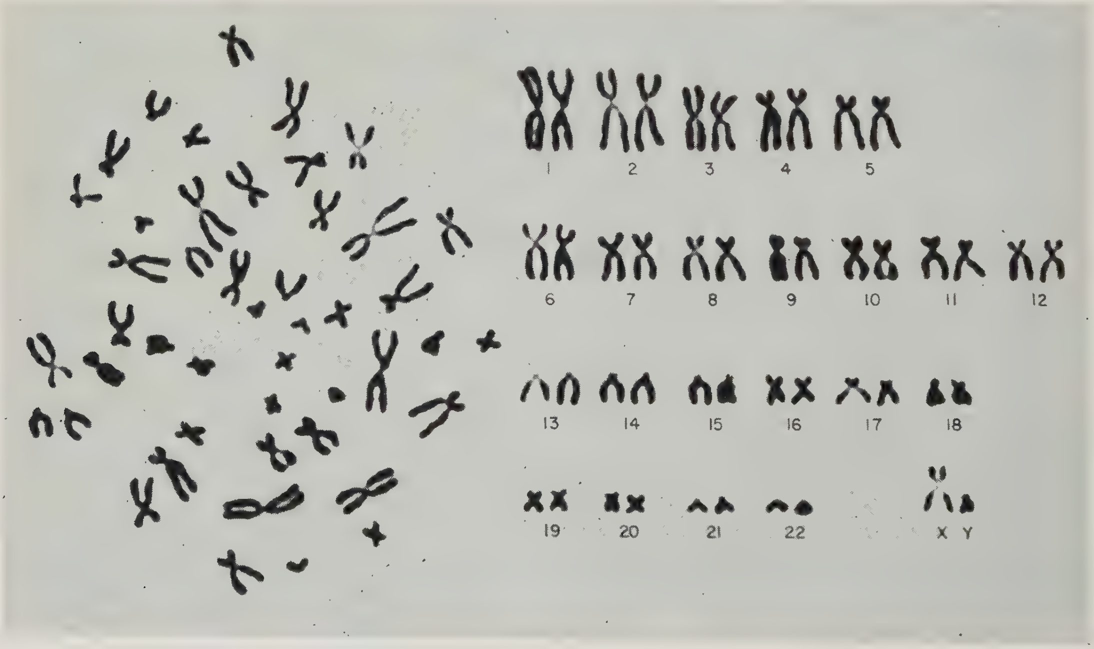


Fig. 338. Left, chromosomes of a dividing human cell at metaphase. Right, karyotype of normal human male showing 22 pairs of autosomes and XY sex chromosomes.

scribed in sexual abnormalities such as hermaphroditism, Turner's syndrome, and Klinefelter's syndrome. They have been found in mongolism, in leukemia, and in congenital defects such as achondroplasia, hemophilia, phenylketonuria, and Marfan's syndrome. Irradiated cells and tumor cells have shown an abnormal chromosomal pattern.

Methods for human chromosome analysis*

Blood culture method

1. Draw 10 ml. of the patient's blood into a sterile heparinized tube and add 0.25 ml. Bacto-phytohemagglutinin.
2. Leave the tube in iced water for 1 hr.
3. Centrifuge at 400 r.p.m. for 10 min.
4. Transfer the supernatant plasma containing the white cells in 3 ml. amounts to several 2 oz. sterile prescription bottles each containing 5 ml. tissue culture fluid.†
5. Incubate the bottles containing the cell cultures on their sides at 37° C. for 3 days. During the last 2 hr. of the incubation period, add Colcemide to a concentration of 1 μg/ml.
6. Transfer the cell culture to a clean dry centrifuge tube and centrifuge for 5 min. at about 1000-2500 r.p.m.
7. Discard the supernatant liquid.
8. Add about 7 ml. 1.12% sodium citrate to the cell deposit.
9. After 15 min. at 37° C. recentrifuge the tube at about 1000-1500 r.p.m.
10. Fix the cell deposit in about 7 ml. 50% acetic acid in water.
11. Leave the cells in this fixative for a few min.
12. Recentrifuge the tube for 5 min. at 1000 r.p.m.
13. Make routine blood films with the cell deposit on clean slides and quickly air dry them.
14. Stain the dried films in 2% aceto-orcein in 65% acetic acid, dehydrate, and mount in the routine manner.

Bone marrow culture technic

Cells suitable for chromosome analysis may be obtained by using a short-term culture technic for bone marrow based on the method of Ford et al.[15]

1. Remove the bone marrow by sternal puncture under local anesthetic and disperse approximately 0.5-1 ml. specimen into each of several sterile tubes each containing 10 ml. heparinized Ringer solution (50 units heparin/ml.).
2. Remove the Ringer solution by gentle centrifugation.
3. Resuspend the cell deposit in 3 ml. of patient's own serum.
4. Transfer this to sterile 2 oz. prescription bottles containing 5 ml. cell culture fluid.*
5. Incubate on their sides at 37° C.
6. It is usually possible to obtain sufficient marrow for several preparations. Incubate the first bottle for 5-7 hr. only. During the last 2 hr. of this period, add deacetylmethylcolchicine (Colcemide) to a final strength of 2 μg/ml. (This drug is thought to interfere with the formation of the mitotic spindle and so serves to arrest and accumulate cells in mitosis.)
7. After incubation, centrifuge the cell culture and resuspend the cells in 10 ml. 1.12% (hypotonic) aqueous sodium citrate for 20-30 min. at 37° C. The hypotonic treatment distends the cells and disperses the chromosomes throughout each mitotic cell.
8. Once again centrifuge the suspension and replace the supernatant fluid by 2 changes of 50% glacial acetic acid.
9. Fix the cells for 15 min. and resuspend in 2 or 3 drops 2% lactoaceto-orcein stain.[16]
10. Make squash preparations from this cell-stain mixture.
11. Treat the remaining bottles similarly on successive days.

Aceto-orcein stain for chromatin patterns

Reagents:

1. Fixative. Rapid fixation in equal parts of ether and absolute ethyl alcohol (EtOH) or in 95% EtOH.
2. Aceto-Orcein stock stain

Orcein	1 gm.
Glacial acetic acid	45 ml.
Distilled water	55 ml.

   (a) Heat the acid on a hot plate to 80°-85° C. Be sure to use asbestos pad.
   (b) Add the orcein, shaking rapidly or using a mechanical stirring rod.
   (c) Gradually add this solution to the distilled water (room temperature), stirring constantly.
   (d) Stopper the flask and bathe in cold running water, shaking to cool the solution.
   (e) Filter through No. 1 filter paper and store in a brown Coplin jar with a screw top. The stain improves with age.

3. Fast green stock stain

Fast green	0.03 gm.
95% EtOH	100 ml.

   (a) Add the fast green to the alcohol and stir to dissolve.

Procedure:

1. Run the slide through 50% EtOH, 5 dips and distilled water, 5 dips.
2. Place the slide in aceto-orcein stock stain for 5 min.
3. Wash for 10 sec. with distilled water in a gentle wash bottle stream.
4. Dehydrate in 50, 70, 80, and 95% EtOH, 5 dips each.

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*These methods, courtesy Dr. M. A. Ferguson-Smith, Division of Medical Genetics, Department of Medicine, Johns Hopkins Hospital School of Medicine, Baltimore, Md., are based on the work of Ford,[15] Welshons and Russell[16] and Ferguson-Smith et al.[17].

†Difco N.C.T.C. 199; Difco Laboratories, Detroit, Mich.

*Difco N.C.T.C. 109; Difco Laboratories, Detroit, Mich.

5. Stain in fast green stock stain for 1 min.
6. Wash in 95% EtOH, 100% EtOH, and 100% EtOH-xylol, 5 dips each.
7. Place in xylol for 5 min.
8. Mount.

FUNCTIONS OF SPLEEN

The general conception of the function of the spleen is that it is a reservoir or bank of blood. In fetal life erythropoiesis begins in the spleen, but the main function of the spleen is that of lymphopoiesis. The spleen is concerned with the destruction of worn-out red cells, leukocytes, and blood platelets, and also assists in the destruction of protozoa and foreign material. The destruction within the sinusoids is brought about by sequestration, i.e., separation, collection, and stasis of the to-be-destroyed tissues. The next step is phagocytosis by the reticuloendothelial system or destruction by lysis. The latter process produces swelling of the affected cells which then rupture and disintegrate. The lytic destruction of red blood cells thus releases iron which is either reutilized or stored in the spleen.

Since the spleen is part of the lymphatic system, it participates with it in the alarm (stress) reaction and in the production of gamma globulins and antibodies. It has been suggested that lack of resistance to infection follows splenectomy. The spleen may also be involved in the production of a coagulation factor of globulin nature.

In fetal life myeloid cells are also produced in the spleen. In the adult, under pathologic conditions, this fetal function is reactivated and gives rise to foci of myeloid metaplasia in the spleen.

This organ contains more endothelial tissue than any other organ of the body. In adult life lymphocytes are produced by the malpighian corpuscles. Many investigators believe that monocytes and reticuloendothelial cells arise in the spleen largely because of its abundant reticuloendothelial structure. It is also probable that the spleen exercises some sort of control over the bone marrow. Splenectomy is often followed by the appearance in the peripheral blood of nucleated red cells, Howell-Jolly bodies, basophilic cells, and reticulocytes, together with an increase in the number of platelets. Following splenectomy, there is also a great increase in the number of leukocytes, platelets, and red blood cells. It is the appearance of Howell-Jolly bodies, however, that is the most characteristic phenomenon following splenectomy.

Not only surgical but also physiologic and pathologic removal of the spleen, as may occur after extensive infarction, hypoplasia, aplasia, or calcification of the spleen, is followed by increases in all blood cellular elements or in 1 or 2 series only.

Whereas splenectomy is at times followed by an increase in all of the cellular components of the peripheral blood, splenomegaly or hyperplasia of the spleen is frequently accompanied by neutropenia, thrombocytopenia, or anemia or by all 3, **splenic pancytopenia.** There is thus some evidence that the normal spleen controls the production of cells in the bone marrow and that the removal of the spleen at times leads to unrestrained proliferation of the marrow elements, whereas hyperplasia leads to splenic hyperfunction and bone marrow depression. The mediating factor is probably humeral in nature and is produced by the reticuloendothelial system. Besides the humeral factor, other elements may play a role. Increased phagocytic activity may be responsible for the production of anemia but may also affect platelets and white blood cells. Also, a hemolytic antigen-antibody mechanism (hemolysins, etc.) may be responsible for decreased survival time of one or all of the corpuscular blood elements, which are thus more rapidly destroyed in the spleen. Finally, the existence of a toxic factor produced in the spleen has been postulated which may be responsible for the bone marrow depression.

It must be stated that splenic changes are not always accompanied by hematologic alterations so that, for instance, posttraumatic surgical removal of the spleen is frequently not followed by any or by only very transient hematologic changes.

FUNCTIONS OF BLOOD

The chief functions of the blood are to transport food materials to the tissues, to carry oxygen from the lungs to the tissues and carbon dioxide from the tissues to the lungs, to carry waste materials from the tissues to the excretory organs, to transport endocrine secretions from one organ to another, to act as a defense mechanism against infection through the activities of certain white cells and immune bodies developed in the blood stream, to assist in maintaining a constant water balance and correct osmotic equilibrium in the tissue cells, and, further, to assist in the maintenance of a constant body temperature and to maintain a degree of irritability of the tissue cells so that functional activity may be carried on satisfactorily.

Chapter 60

Composition and physiology of the blood

JOHN D. BAUER

The circulating blood normally consists of a suspension of blood cells of different kinds in a fluid element called plasma. The formed elements are erythrocytes, leukocytes, and blood platelets. Plasma is the liquid portion of blood of a very complex composition: water, proteins, carbohydrates, lipids, electrolytes, other mineral salts, hormones, antibodies, and enzymes. If blood is allowed to clot, fibrinogen is removed from the plasma, and the remaining fluid is called blood serum.

The total volume of the blood comprises about 6 to 8% of the body weight in adults.

There are normally from 4,600,000-6,200,000 red blood cells/c.mm. in the adult male (average 5,400,000) and from 4,200,000-5,400,000 in the adult female (average 4,800,000). There are from 5,000-10,000 leukocytes normally, with an average of 7000-8000/c.mm. The number of blood platelets varies from 200,000 to 300,000/c.mm. of blood according to some authorities, and is higher in men than in women and children.

RED BLOOD CELLS OR ERYTHROCYTES

The normal red blood cell or erythrocyte is a circular to slightly oval biconcave disc with an average diameter of 7.2μ. It is orthochromatic. It is bright yellow with a central depression, is highly light-refractile, and apparently is homogeneous. The red cell in the circulating blood is normally nonnucleated. Red cells originate in the bone marrow. Jordan and Baker held that the red cells are released into the circulation through gaps in the endothelial walls of the capillaries.

Table 224 includes some of the attributes of red blood cells.[18]

Table 224. *Physical measurements of red blood cells*

Red blood cells	*Average*	*Range*
Diameter	7.5μ	7.2-7.8μ
Thickness	2.0μ	1.7-2.2μ
Volume	87.0 c.μ	70-94 c.μ
Hemoglobin content	29 $\mu\mu$g	23-35 $\mu\mu$g

The biconcave shape is partly determined by the tension of the cell membrane and partly by the cell contents. The presence of abnormal hemoglobins may change the shape of the red cells. It is the stroma (structural protein) to which the hemoglobin is attached and which forms the elastic, selectively semipermeable cell membrane.

FATE AND PHYSIOLOGY OF RED BLOOD CELLS

Many different methods have been used to estimate the average life span of the human red blood cell. Callender et al.,[19] using Ashby's differential agglutination method[20, 21] in Rh-positive men,[22] concluded from a mathematical analysis of their data that red blood cells live for approximately 120 days. Measurement of the time required for the disappearance of sulfhemoglobin from the blood of cyanosed workers[23] indicated the life span of the red blood cells to be 115 days. Shemin and Rittenberg[24] reported their results in estimating the life span of the red blood cell by estimating isotope concentration of the hemin at various periods in experimental works. They showed that the heme of the nonnucleated red blood cells, unlike the constituents of nucleated cells, is not continuously formed and degraded within the cell. The red cells are not indiscriminately destroyed, but their rate of destruction is a function of the age of the cell. Study of the isotope concentrations found in the heme of the human red blood cell, after the feeding of glycine labeled with N^{15}, indicated that the erythrocyte is not subjected to indiscriminate destruction but has a life span. This was found to be about 127 days.

If it is assumed that the life span is 120 days, and if the estimate is 5,000,000 red cells/c.mm. of blood, the bone marrow would have to manufacture about 250 billions of red cells every day. This means a production of 42,500 red cells for each c.mm. of blood each day. About 0.83% of the red cells and hemoglobin are replaced daily.

Returning to the question of the origin of erythrocytes, according to the Schilling idea, the formation of these cells begins as indifferent erythrogonia, followed by the proerythroblast with nucleoli, then by typical hemoglobin-containing macrocytic erythroblasts, and then polychromatic and orthochromatic smaller normoblasts. Then, in turn, come the denucleated polychromatic cells and, finally, the full-grown orthochromatic normocytes. There are many explanations of the denucleation of the erythrocyte. First, Maximow claimed that the expulsion is seen in the embryonic body although this is denied by Schridde. Albrecht is in favor of the expulsion theory. A second theory is that of karyorrhexis, in which the pyknotic nucleus assumes bizarre shapes and is dissolved into spherical sections, leaving the cell gradually. Weidenreich[25] maintained that karyorrhexis may go on to further reduction, giving the appearance of small chromatin rodlike changes. Next, there is a theory of intracellular nuclear disintegration, which is largely the belief of Naegeli.

It is interesting to note the remarkable amount of basophilic substance in the young forms of the erythrocytes and also to note when hemoglobin is first seen in embryonic blood. Wulf claims to have seen traces of hemoglobin by means of the spectroscope in the embryo with 9 primary segments. The hemoglobin shows up first in the mitochondria, which are found in the youngest cell. In this connection, study of polychromasia by supravital staining methods shows that it may degenerate into basophilic punctation. In other words, polychromatic vital-staining reticular substance and basophilic punctation are protoplasmic and not nuclear phenomena. Normally, polychromasia is seen in peripheral blood in the proportion of 0.2-1% of all red cells. Pathologically, this may range as high as 50%, as seen in the anemias and the after effects of acute infections, of malaria, and of tuberculosis, in blood crises of pernicious anemia, and in hemolytic icterus.

Erythropoiesis

Physiologic processes which may affect erythropoiesis are menstruation and pregnancy, hemorrhage, and blood destruction. There is a rapid production of red cells after venesection, first, in the form of migrating maturing forms and, second, as nucleated elements, immature polychromatic erythroblasts, and normoblasts. At this time, liberation of many young forms takes place. Any decrease in the quantity of erythrocytes is regarded as a physiologic stimulant to the formation of more erythrocytes. However, daily blood transfusions in quantity equal to the destroyed blood lead to inertia of the destroyed bone marrow and aplasia. A single injection of a physiologically destroyed amount of erythrocytes results in immediate irritation of the bone marrow. This irritation may be followed within 4-7 days by prolonged increased erythropoiesis. Lack of oxygen may also be an effective physiologic stimulant, such as is seen in high altitudes. Increase in the number of erythrocytes due to chronic lack of oxygen is seen in heart diseases, congenital pulmonary stenoses, and mediastinal tumors. Hypernephromas, cerebellar tumors, hemangioblastomas, and ovarian tumors are known to be associated with polycythemia.

The humeral control of erythropoiesis is generally accepted. Linman and Bethell[26] described 2 erythropoietic plasma factors which differ chemically and physiologically. One factor, a nondialyzable mucoprotein which is relatively thermolabile and ether insoluble, is apparently responsible for the initial proliferation of the hematocytoblast or proerythroblast to differentiate and to form hemoglobin. This factor is called **erythropoietin.** It appears to depend upon good kidney function. The second plasma factor, thermostable and ether soluble, is not a protein and is responsible for the second phase of erythropoiesis, the proliferation of normoblasts within the bone marrow and is found in patients with primary and secondary polycythemia. The first factor essentially controls the hemoglobin synthesis, whereas the second factor increases the number of red blood cells without increasing hemoglobin formation. The second factor appears to be independent of kidney function.

Hypoxia (or the relationship of oxygen supply to tissue requirements) is the main stimulus to erythropoiesis. How and where hypoxia or any other erythropoietic stimulus acts is not certain. Experimentally, the bone marrow, stomach, kidney, liver, and spleen can either be ruled out as the site of production or can be used to prove that there are several types of erythropoietic substance. This substance can be found in the urine of severely anemic patients, and there is increasing evidence, as already stated, that intact kidney function is related to erythropoiesis.

Erythropoiesis proceeds according to a regular predetermined pattern and rhythm in erythroblastic nests within the bone marrow, forming what has been called **living erythrons.** The youngest early precursor cell (proerythroblast) divides hemihomoplastically, giving rise to 2 smaller erythroblasts, one of which again forms an early erythroblast, whereas the other divides progressively into 2, 4, and 8 normoblasts. On the basis of nuclear volume measurements of the cells of the erythron, Weicker assigned a given nuclear volume to each cell type and originated the karyometric method of cell classification (p. 1068).

Functions of erythrocytes

The physiologic functions of the erythrocytes include (1) gas exchange, the transport and release of oxygen and of carbon dioxide, (2) participation in the buffer system of the blood, and (3) a role in the clotting mechanism, according to Quick, mentioned in the discussion on coagulation.

Respiration

The red blood cell accepts oxygen and releases CO_2 in the lung capillaries. It then transports the gases from the lung to the tissues. Transport is made possible by the ability of hemoglobin to combine reversibly with oxygen, as represented by the following equation:

$$\text{Hb} + \text{O}_2 \rightleftarrows \text{HbO}_2$$

Hb = reduced hemoglobin
HbO_2 = oxyhemoglobin

The degree of saturation of hemoglobin with oxygen depends on the CO_2 tension which can be shown to determine the shape of the dissociation curve. If the oxygen tension is high ($p - O_2$ 100 mm. Hg as it is in arterial blood), hemoglobin is about 95% saturated. If the oxygen tension falls, oxygen is rapidly released. In addition to the CO_2 tension, the dissociation curve is influenced by such factors as temperature and electrolytes. Oxygen is carried only by the intracellular hemoglobin, whereas carbon dioxide is carried by hemoglobin and plasma.

Buffering action

Oxyhemoglobin is a stronger acid than reduced hemoglobin. At a constant pH, a larger amount of alkali is necessary to neutralize oxyhemoglobin than reduced hemoglobin. When oxygen is released at the tissue level, alkali is also released to neutralize the carbonic acid formed in the interstitial fluid in the presence of CO_2.

In the lung, where CO_2 is given off, the freed alkali is taken up again by hemoglobin. The intracellular hemoglobin is responsible for 60% of the buffering ability of the blood. This result is achieved by the **chloride shift,** which allows chloride to leave the red cell whenever the CO_2 tension falls and allows bicarbonate to enter the cell. This is a reversible process so that chloride enters the cell when the CO_2 tension increases, and bicarbonate is released in exchange for chloride (Hamburger phenomenon).

The chemical composition and metabolism of the red cell have important bearing on various blood diseases.

The cell membrane is impermeable to sodium and potassium but allows the entry and release of hydrogen, bicarbonate, and chloride ion. The erythrocyte, therefore, contains more potassium than sodium. The retention of potassium represents a vital, energy-requiring process of the cell utilizing glucose as an energy source. The enzyme system acetylcholine cholinesterase I functions as a guard against changes in permeability of the cellular membrane.

In the breakdown of glucose, the first step is the formation of glucose 6-phosphate. In the course of aerobic glycolysis, this product is broken down to citric acid which is degraded to carbon dioxide in the Krebs cycle. The anaerobic glycolysis which provides the main source of energy metabolizes glucose 6-phosphate to pyruvic and lactic acids (Embden-Meyerhof scheme). Pyruvic acid is decarboxylated to acetic acid which enters the citric acid cycle of Krebs. In anoxemic newborn infants and in premature babies the complete breakdown of lactic acid to carbon dioxide and water is often not accomplished so that the accumulated lactic acid leads to acidosis. An alternate pathway of the metabolism of glucose 6-phosphate is the direct oxidation to 6-phosphogluconate by the action of glucose 6-phosphate dehydrogenase. Deficiency of this enzyme leads to defective direct oxidation of glucose in red cells and to such phenomena as Heinz body formation and primaquine type of hemolytic anemia.

Hemolysis

The factors controlling hemolysis are poorly understood but are related to the activity of the reticuloendothelial system. The mechanisms of hemolysis will be considered in the discussion of hemolytic anemias.

HEMOKONIA

In the dark-field preparation, one finds **hemokoniae,** which are fine dustlike particles with brownian movement. Neumann[27] considers these bodies fatty particles and notes that they are prevalent particularly after eating. They are not cells but may be degradation products of cells.

HEMOGLOBIN

Hemoglobin is the red iron-bearing protein contained within the erythrocytes in normal blood. Its major function in the normal individual is as a carrier of oxygen from the lungs to the tissues where it readily releases this oxygen to the tissues and then returns to the lungs to combine with more oxygen. This respiratory function of hemoglobin is vital to the life of all cells. Although not all of the physicochemical aspects of hemoglobin have been established, certain basic facts are generally accepted: Hemoglobin consists of 4 heme molecules conjugated with 1 globin molecule. The heme molecule represents the colored component of the hemoglobin molecule. The globin fraction is a complex protein with a molecular weight between 63,000 and 67,000. The source of globin is protein in the diet, which is broken down in the gastrointestinal tract to amino acids of various types, which in turn are absorbed and used in hemoglobin synthesis. Although some of the amino acids found in globin have been identified as arginine, histidine, lysine, tryptophane, and tyrosine, it is believed that no one single amino acid is alone essential for hemoglobin synthesis. Orten and Orten[28] have demonstrated that, if any one of the essential amino acids is added to the diet, there is no increase in hemoglobin synthesis. The amount of dietary protein required to keep the body in satisfactory equilibrium so far as hemoglobin synthesis is concerned is approximately 1 gm./kg. body weight. Whipple,[29] in his classical experiments, demonstrated that if protein is not obtained from outside the body in the diet, then the body will selectively take protein from tissue in order to synthesize hemoglobin: "the body guards jealously the fabrication of the hemoglobin, and given a need for both plasma protein and hemoglobin, the protein flow favors hemoglobin synthesis."* Practical use of this was made in the care of the wounded under the program known as Reparative Surgery during World War II.[30-34]

Not only will inadequate food intake affect the amount of hemoglobin formed, but also poor absorption of this protein from the gastrointestinal tract will affect the amount of hemoglobin absorbed; therefore, such gastrointestinal affections as protracted diarrhea in colitis, atrophic gastritis, and other conditions will result in hemoglobin deficiency. There is present in the gastrointestinal tract intrinsic factor and erythrocyte maturation factor which affect hemoglobin formation. In some unknown way these factors are related to the fabrication of hemoglobin through the intermediary role of the liver prior to the taking up of the substances in the bone marrow by the red blood cells.

Variations in the composition of the globin component are responsible for the appearance of abnormal human globins. The protein composition is genetically determined, and these changes are part of the expressions of gene mutations. The heme component is the same in all hemoglobin. (For details see discussion of abnormal hemoglobins.)

The 4 heme molecules of hemoglobin consist of 4 protoporphyrin molecules containing ferrous iron. Although iron is important in the synthesis of hemoglobin, exceedingly small amounts are required in the diet for normal hemoglobin synthesis.

Hemoglobin consists of 4 heme molecules chemically identified as ferrous protoporphyrins. The chemical alignment of these porphyrins in relation to globin in the formation of hemoglobin has been extensively studied by Pauling.[35]

The protoporphyrin is formed in the bone marrow erythroblasts out of glycine and succinate. Though the biosynthesis of porphyrin will be discussed later, it should be mentioned that one of the intermediary products, delta-aminolevulinic acid, is also used for the synthesis of vitamin B_{12}. Vitamin B_6 and panto-

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*From Whipple, G. H.: Am. J. M. Sc. **203**:477, 1942.

thenic acid are required for the production of delta-aminolevulinic acid.

The iron required for this hemoglobin synthesis from the **diet alone** is almost infinitesimal, being perhaps approximately 12 mg. as the daily average minimum requirement. This amount is readily available from any average diet; it requires an unusually subnormal diet to bring iron intake to levels lower than this. The reason for this is that the iron stored within the body is not depleted unless there is loss through recurrent hemorrhage or a great increase in demand such as occurs in early childhood, during the growing period, and in pregnancy. When extra iron is needed, it may be given in the form of iron salts which are absorbed from the gastrointestinal tract in the ferrous state. Under unusual circumstances, parenteral iron therapy may be necessary to supply the depleted iron stores of the body.

Iron absorption is aided by reducing substances such as ascorbic acid. The role of hydrochloric acid is not quite clear though studies by Moore fail to show that ionization of iron produced by hydrochloric acid has any stimulating effect on iron absorption. The iron absorption is influenced by the state of the iron stores, and the rate of erythropoiesis. Iron absorption is increased if the iron stores are reduced and if erythropoiesis is stimulated. Iron metabolism is considered in detail in the discussion of iron deficiency anemia.

Iron combines in the small intestine with a protein, forming **ferritin.** It is then released from the mucosal cells of the small intestine to the plasma, where it has been shown that β_1 globulin carries the iron in combination known often as **transferrin.** Associated with this globulin, it is transported to the bone marrow where the iron combines with protoporphyrin to form heme. The iron is transferred from reticulum cells to erythroblasts by the process of pinocytosis. The heme then combines with globin to form hemoglobin.

Breakdown of hemoglobin

If hemoglobin is liberated from the red cells, it is immediately broken down. Normally, hemoglobin is broken down in the reticuloendothelial system where the heme portion of the hemoglobin molecule is transformed to nonconjugated bilirubin which is ultimately conjugated by the liver and excreted. The iron is retained and reutilized for the production of hemoglobin and other iron-containing porphyrins such as cytochromes and myoglobins. The globin moiety is also retained and split into its amino acid components, which are reutilized.

Intravascular lysis, which is usually a pathologic process, produces free hemoglobin which unites with haptoglobin and either remains in the serum, or, if the concentration is high, is excreted by the kidney. Some of it is also taken up by the reticuloendothelial system and is converted to free bilirubin.

The breakdown of hemoglobin is considered in detail in the discussion of hemolytic anemias.

Hemoglobin normally is in the state in which it has absorbed oxygen and is known as **oxyhemoglobin.** Oxyhemoglobin can be identified in the spectroscope by the 2 intense absorption bands at 578 and 540 mμ.

Technic of spectroscopic examination of hemoglobin:

1. Dilute 1 part blood or washed red cells with 100 parts water or with 100 parts 1% sodium hydroxide.
2. Observe 2 absorption bands through the spectroscope; 1 band will be in the yellow zone and 1 in the green zone.
3. Addition of a few crystals of potassium ferrocyanide converts oxyhemoglobin into methemoglobin which has only 1 absorption band at 630 mμ.
4. Addition of ammonium sulfide to the original oxyhemoglobin solution produces reduced hemoglobin with only a single band in the yellow and green zones.

Abnormal forms of hemoglobin

Methods for the determination of hemoglobin are described in Chapter 61.

Methemoglobin[36, 37]

Methemoglobinemia is a disease characterized by a temporary or permanent increase in methemoglobin in the red blood cells. Clinically it leads to cyanosis since there is interference with the oxygen transport. If over 1.7% of the total hemoglobin is present as methemoglobin, cyanosis will occur. Clinically, this cyanosis must be differentiated from the cyanosis produced by acquired or congenital heart and lung diseases (anoxia) and by polycythemia. Unless a hemolytic process is present, methemoglobin is always confined to the red blood cells.

There are 2 types of methemoglobinemia: acquired and congenital (hereditary).

Acquired methemoglobinemia is usually associated with intoxication with chlorates, nitrites, nitrobenzol, aniline, hydrogen sulfide, and other chemical agents. It may be caused by exposure in industry to excessive amounts of these agents or may result from excessive intake of drugs, or it may result from idiosyncrasies to certain drugs, such as acetanilid and sulfanilamide.

Methemoglobinemia is also caused by overproduction or excessive absorption of nitrites which may be derived from the intestines in constipation or diarrhea of long duration, or these nitrites may be contained in the drinking water obtained from a well. Acquired methemoglobinemia is frequently associated with sulfhemoglobinemia due to the fact that intestinal bacteria may produce a large amount of hydrogen sulfide, some of which may be absorbed.

The relationship of methemoglobin to Heinz body formation will be considered in the discussion of Heinz bodies. As methemoglobinemia is apparently a cause of abortion in cattle, some attempts have been made to prove its responsibility for some cases of human abortion.

In hemolytic anemias, methemoglobin may be present in the plasma, usually combined with methemalbumin. This form of methemoglobinemia is considered in the discussion of hemolytic anemias.

Congenital methemoglobinemia, a hereditary disease, has all the characteristics of methemoglobinemia—cyanosis varying in shade and intensity and methemoglobinemia—and in addition a hereditary background. It is inherited as a recessive character and is due to the absence of intraerythrocytic enzymes which mediate the reduction of methemoglobin. The enzyme involved is probably coenzyme factor I (diaphorase I). Methemoglobinemia is thus essentially a mendelian recessive enzyme deficiency disease. It is at times combined with a dominant type of molecular hemoglobin abnormality, hemoglobin-M disease, which, as all the hemoglobinopathies, involves the protein moiety of the hemoglobin molecule.

Laboratory test for methemoglobinemia—spectroscopic examination:

1. Dilute 1 part blood or washed red cells with 100 parts distilled water or with 100 parts 1% sodium hydroxide. Examine without delay because methemoglobin may be formed spontaneously on standing. Examine with the spectroscope. If **methemoglobin** is present, a band will appear at 630 mμ. **Sulfhemoglobin** will produce a single band which with the usual spectroscope cannot be distinguished from the methemoglobin band.
2. Add a few drops 5% solution potassium cyanide, and if the band is due to methemoglobin, it will disappear. The sulfhemoglobin band will remain.
3. Add 2 or 3 drops 3% hydrogen peroxide. Methemoglobin or sulfhemoglobin bands will disappear.

Methemoglobin in acquired methemoglobinemia quickly disappears from the circulation after exposure to the causative agent has ceased.

Carboxyhemoglobin

Carboxyhemoglobin (carbon monoxide hemoglobin) results from a combination of hemoglobin and carbon monoxide. It has been claimed that hemoglobin has 200 times the affinity for carbon monoxide that it has for oxygen. Carboxyhemoglobin is found in the blood after exposure to inhalation of artificial gas, automobile exhausts, underground gas collections, or other agents containing carbon monoxide. It is believed that life can be maintained with a 20% concentration of carbon monoxide hemoglobin, but that a concentration of 50% or greater will result in death.

Carboxyhemoglobin may be identified by spectroscopic examination. It must be remembered that the bands are at 572 and 535 mμ, which are very close to those of oxyhemoglobin. Sunderman has suggested the following spectroscopic test.

Sunderman dithionite spectroscopic test:

1. Dilute the blood with water until the 2 bands in the spectrum of both carbon monoxide hemoglobin and oxyhemoglobin are still clear but faintly visible.
2. Add 5 mg. dithionite ($Na_2S_2O_4$).
3. Mix gently and examine for persistence of the bands of carboxyhemoglobin. If 2 bands persist, carboxyhemoglobin is present.

Other tests are given in the section on Toxicology.

Sulfhemoglobin

Sulfhemoglobin is an abnormal hemoglobin, the exact chemical structure of which is unknown. Sulfhemoglobinemia often accompanies methemoglobinemia. Sulfhemoglobinemia has been described in patients who habitually take alkaline bromides as a headache remedy and in others who have either been exposed to large doses of phenacetin or sulfonamides or have idiosyncrasies to these drugs.

The differentiation of methemoglobin and sulfhemoglobin is considered in the discussion of methemoglobinemia. It has been stated that methemoglobin quickly disappears from the circulation after exposure to the causative agent has ceased, whereas sulfhemoglobin persists for several weeks and is destroyed at the same time as the involved red blood cells.

Sulfhemoglobin shows a main spectroscopic absorption band at 618 mμ. It is found in both the red blood cells and the plasma. Unlike methemoglobin, addition of 5% cyanide (KCN) will not cause the band to disappear. Sodium dithionite ($Na_2S_2O_4$) also will leave the band unchanged. Sodium sulfite and carbon monoxide will leave the band unchanged, but sodium sulfite with sodium hydroxide added causes disappearance of the band as does 3% hydrogen peroxide.

The inherited abnormalities of the hemoglobin molecule are discussed in Chapter 64.

Myoglobin

Myoglobin or **myohemoglobin** in the urine has been described in relation to muscle injury. Detection of myoglobin in the urine must be made within 8-12 hr. after the injury since the pigment tends to disappear after this time. Myoglobin is excreted by the kidneys at the slightest increase of its plasma level. In contrast to hemoglobin, myoglobin is not bound to haptoglobin, but it may be bound to some other serum protein.

Tests for myoglobinuria[38]

Spectroscopic examination

Sunderman and associates recommend examining urine for absorption bands at 582 and 542 mμ. Since these bands are very close to those of oxyhemoglobin, it is impossible to make a positive diagnosis by the spectroscope alone though fluorescent light is helpful.

Dithionite test

Add 10 mg dithionite to 10 ml. urine and place in a 9 collodion filter.*

If hemoglobin is present, a clear filtrate is obtained, whereas in the presence of myoglobin a brown filtrate with an absorption band at 583 mμ is obtained.

Differential solubility test

At 80% saturation with ammonium sulfate hemoglobin in solution is completely precipitated from the solution along with denatured myohemoglobin, but only minor amounts of undenatured myohemloglobin are included. At 100% saturation myoglobin is completely precipitated together with normally occurring urinary pigments. Prior to the test, the protein nature of the dissolved hemoglobin or myoglobin is to be proved by the sulfosalicylic acid test. Both these pigments also give the benzidine reaction.

Procedure:

1. To 1 ml. urine add 3 ml. 3% sulfosalicylic acid. Mix and filter. If the pigment is precipitated it is a protein.
2. To 5 ml. urine add 2.8 gm. ammonium sulfate and mix. Filter. If the filtrate is of abnormal color, myohemoglobin is present.

PORPHYRIA AND PORPHYRINURIA[39, 40]

Hemoglobin, as already stated, is an iron porphyrin because an iron atom is incorporated into the porphyrin structure. A porphyrin consists of 4 pyrrole rings linked to each other by methylene bridges. Each porphyrin has 8 substituent positions which allow the attachment of side chains. The type and symmetry of arrangement of the side chains distinguish the various types of porphyrins. Hemoglobin synthesis is based on a complete synthesis of protoporphyrin. Not all porphyrin is used in hemoglobin production so that a small amount of free porphyrin and coproporphyrin can be found within the red blood cells. This substance is called free erythrocyte protoporphyrin and coproporphyrin.

Normal values of protoporphyrin in mature erythrocytes range from 20-50 gamma/100 ml. cells. These values are increased to 150-350 gamma in lead poisoning, cirrhosis, iron deficiency, and congenital porphyria; they are decreased or normal in pernicious anemia. Red blood cells containing much erythrocytic protoporphyrin fluoresce under ultraviolet light. Free coproporphyrin in the red blood cell corresponds fairly closely to the reticulocyte level, which, as will be seen later, is a good indicator of red cell maturation or hemoglobinization.

Biosynthesis of porphyrins

Glycine plus active succinate derived from Krebs tricarboxylic acid cycle or from DPN give rise to delta-aminolevulinic acid. This production is dependent on an adequate supply of pyridoxine. Two molecules of delta-aminolevulinic acid form porphobilinogen. This process is catalyzed by copper-containing enzymes. Porphobilinogen is then converted into uroporphyrinogen III. The latter substance is enzymatically transformed to coproporphyrinogen III. An enzyme converts

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*Gradacol Elford.

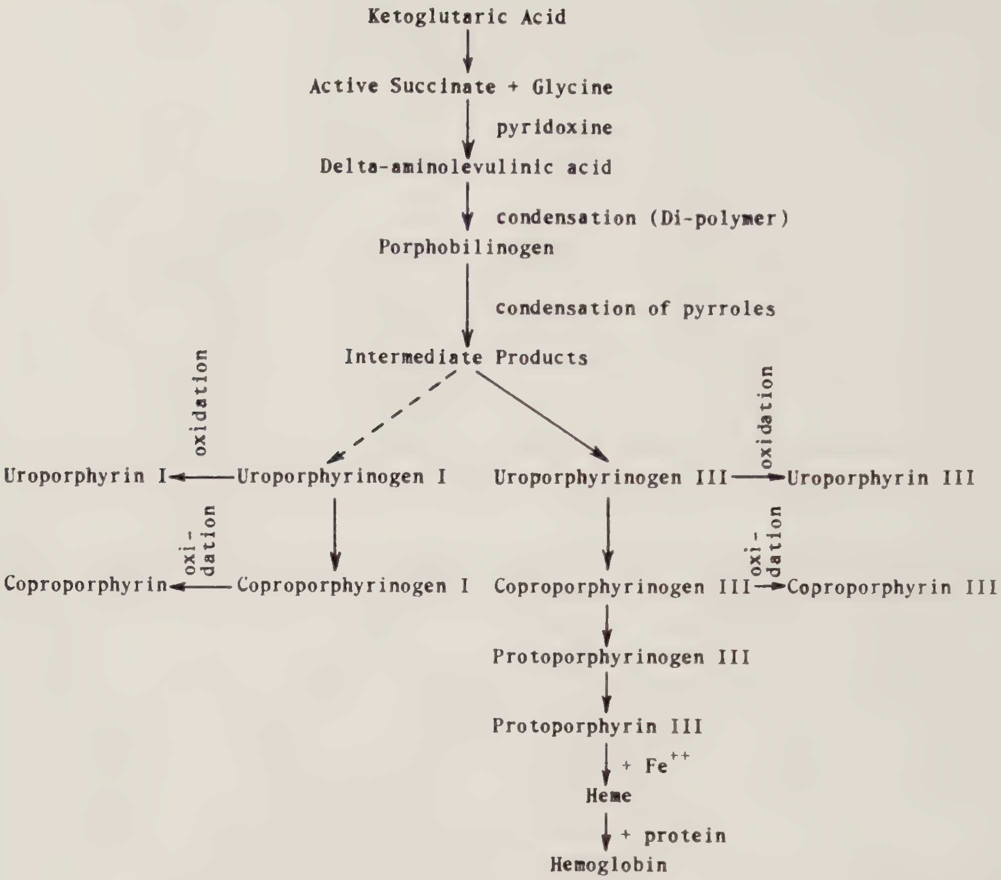
Ketoglutaric Acid
Active Succinate + Glycine
pyridoxine
Delta-aminolevulinic acid
condensation (Di-polymer)
Porphobilinogen
condensation of pyrroles
Intermediate Products
oxidation
Uroporphyrin I
Uroporphyrinogen I
Uroporphyrinogen III
oxidation
Uroporphyrin III
oxi-dation
Coproporphyrin
Coproporphyrinogen I
Coproporphyrinogen III
oxi-dation
Coproporphyrin III
Protoporphyrinogen III
Protoporphyrin III
+ Fe++
Heme
+ protein
Hemoglobin

Fig. 339. Outline of porphyrin synthesis. (Based on data from Harper, H. A.: Review of physiological chemistry, Los Altos, Calif., 1961, Lange Medical Publications; from Bauer, J. D., Toro, G., and Ackermann, P. G.: Bray's clinical laboratory methods, St. Louis, 1962, The C. V. Mosby Co.)

coproporphyrinogen III to protoporphyrin. In the production of heme, ferrous iron is incorporated into protoporphyrin. The term **porphyrinogen** is used to describe the reduced forms which can be oxidized to porphyrins. The term **type III** indicates a porphyrin with asymmetrical substitution, whereas the term **type I** characterizes a porphyrin with symmetrical arrangement of the substituents. These substituents are in the case of uroporphyrins acetate and proprionate and in the case of coproporphyrins methyl groups and proprionate. Under circumstances which are as yet not well understood, enzymatic transformation of porphobilinogen into uroporphyrin I occurs which then gives rise to coproporphyrin I.

The term **porphyria** embraces certain hereditary and familial conditions in which there is an increased excretion of coproporphyrins and uroprophyrins in urine, feces, or both.

The term **porphyrinuria** includes many conditions such as poisoning by heavy metals, blood diseases, etc., which lead to an increased excretion of coproporphyrins in the urine.

Porphyrias reported from South Africa and Turkey that are clinically and biochemically reminiscent of the congenital type of porphyria but that are, in fact, due to exposure to poisons made it advisable to review the classification of porphyrias.

Watson suggested the following classification of porphyrias.

Classification of porphyrias (Watson)[41]

I. Porphyria erythropoietica (recessive)
II. Porphyria hepatica
    A. Hereditary acute intermittent (dominant)
        1. Manifest
        2. Latent
    B. Hereditary mixed or variegate group (dominant)
        1. Cutaneous with little or no acute manifestations

  2. Acute intermittent without cutaneous symptoms
  3. Various combinations
  4. Latent

C. Hereditary cutaneous

D. Constitutional or idiosyncratic (porphyria cutanea tarda)
  1. Chemicals, especially alcohol
  2. Idiopathic
  3. With systemic diseases

E. Acquired
  1. Secondary to hepatoma
  2. Secondary to fungicides as in Turkish epidemic

Characteristics of some porphyrias

Porphyria erythropoietica: This is a mendelian recessive and is characterized by light sensitivity. Uroporphyrin I and coproporphyrin I are produced in excessive amounts. The defect may lie in the enzymatic transformation of porphobilinogen. Porphyrin is increased in the marrow, which is the site of the metabolic defect.

Porphyria hepatica:

Hereditary acute intermittent: This is a familial disease characterized by excessive amounts of porphobilinogen, uroporphyrin III, and coproporphyrin III in the urine. The genetic defect may lie in an enzyme system of the liver, involving the production of aminolevulinic acid although an added nongenetic defect may determine the exact nature of the released porphyrin.

Hereditary mixed or variegate type: This disorder is hereditary and nonsex-linked. During acute attacks there are increased porphobilinogen and increased porphyrins which may be uroporphyrin or coproporphyrin type I or type III. There may or may not be light sensitivity.

Hereditary cutaneous: In this disorder there is light sensitivity, and urinary porphyrins, porphobilinogen, and delta-aminolevulinic acid are increased. Fecal porphyrins are not increased.

Constitutional or idiosyncratic porphyria (porphyria cutanea tarda): Excessive amounts of porphobilinogen, coproporphyrin, and uroporphyrin are eliminated in the stool, with bile. During attacks, coproporphyrins and uroporphyrins appear in the urine, but there is little porphobilinogen in the urine.

Acquired porphyria: Acquired porphyria is characterized by coproporphyrinuria due perhaps to an enzymatic defect that affects incorporation of iron into protoporphyrin.

Quantitative estimation of porphobilinogen and porphyrins in urine*

Reagents:

1. Anhydrous sodium carbonate.
   5 gm. in container for collection of 24 hr. urine specimen.
2. Saturated sodium acetate.
   60 gm. sodium acetate in 60 ml. boiling water.
3. Ehrlich reagent.

p-Dimethylaminobenzaldehyde .	0.7 gm.
Concentrated HCl	150 ml.
Distilled water	100 ml.

4. Buffered acetic acid solution.
   1 part of saturated sodium acetate to 4 parts glacial acetic acid.
5. Ethyl acetate, N.F.
6. Sodium acetate, 3%.
   30 gm. sodium acetate/L. of aqueous solution.
7. Alcoholic iodine solution, 1%,
   1 gm. iodine crystals/100 ml. 95% alcohol. Keep in a refrigerator in brown bottles.
8. 1.5N HCl (5%).
   5 ml. concentrated hydrochloric acid in water and diluted to 37 ml. with water.
9. Butyl alcohol. Normal, A.R.
10. Aluminum oxide.
    Most lots are satisfactory, but each lot should be tested to make sure it absorbs uroporphyrins since occasionally a lot is found which will not do so.
11. Acetone, G.P.
12. 50% acetone in 1% acetic acid.

Acetone	250 ml.
Water	245 ml.
Concentrated glacial acetic acid	5 ml.

13. Glacial acetic acid, A.R.
14. Acetic acid, 1%.
    1 ml. glacial acetic acid q.s. to 100 ml. with water.
15. Coproporphyrin standard.
    10 μg/100 ml.

Collection of specimen: Collect a 24 hr. urine specimen in a bottle containing 5 gm. sodium carbonate. Upon receipt, measure the urine volume and record the quantity.

Measure and record the pH of the specimen. If it is not above pH 6.0, the specimen may be processed, but a note should be made in the final report to the effect that the urine was not alkaline upon receipt. It is the function of the sodium carbonate in the collection bottle to keep the urine at an alkaline pH in order to prevent destruction of porphyrins and to inhibit the conversion of porphobilinogen to porphobilin since the latter will give a negative reaction with Ehrlich aldehyde reagent.

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*Courtesy James N. Patterson, Charles Catanzaro, and Doris M. Dede and the Commission on Continuing Education, American Society of Clinical Pathologists, Annual Meeting, Chicago, 1959. These methods are modifications of the technics of Watson and Schwartz. The procedures were adapted to be of practical utility in clinical laboratories in which elaborate equipment may not be available.

Apparatus:

1. Coleman* catalog No. 12-190 photofluorometer cuvets.
2. Coleman model 12C photofluorometer.
3. Coleman filters, catalog No. 12-224 (primary) and No. 14-220 (secondary).
4. Coleman spectrophotometer or Galv-O-Meter.
5. Coleman catalog No. 12-057 connecting cable.
6. Separatory funnel, 250 ml.
7. Graduates, 50 ml. and 500 ml.
8. Chromatograph tube, No. C-4080 (size 2, 19 × 200 mm.).†
9. Suction flask, 500 ml.
10. Sea sand, washed.
11. Portable ultraviolet inspection lamp.
12. Pipets calibrated, 5 ml.
13. Pipets, transfer, 0.1 ml.

Determination of porphobilinogen

Principle: Porphobilinogen is the monopyrrolic precursor of uroporphyrin, coproporphyrin, and protoporphyrin. It reacts with Ehrlich aldehyde reagent (paradimethylaminobenzaldehyde) to form a red color.

Porphobilinogen should be tested for in a freshly voided urine specimen (when available) and also in a 24 hr. specimen. The fresh specimen should first be cooled to room temperature in order to prevent the so-called warm aldehyde reaction, giving a false positive result.

Procedure:

1. Into a No. 6-304 (19 × 150 mm.) Coleman cuvet measure the following; then mix and observe.
   (a) 2.5 ml. urine.
   (b) 2.5 ml. Ehrlich aldehyde reagent.

If porphobilinogen is present, a pink to red color will usually appear at this stage.

2. Add 5 ml. saturated solution sodium acetate and shake. This reagent will convert the hydrochloric acid present in Ehrlich reagent to a weak acid, and when tested with Congo red paper, the paper will remain red. At this point any red color formed in step b above may deepen in intensity. If the color produced is very intense the test should be repeated using 2.5 ml. **quantitatively water-diluted** specimen of urine. Ordinarily, any color due to urobilinogen does not develop until the sodium acetate is added.
3. Add 5 ml. butyl alcohol and shake vigorously. Butyl alcohol will rise to the top and carry with it urobilinogen and various nonspecific Ehrlich-reacting substances, leaving the porphobilinogen in the lower aqueous layer. If the separation is not clear-cut on standing, filter through a moistened coarse filter paper.
4. *Interpretation:* The absence of a pink or red color in the aqueous (lower) phase constitutes a negative test for porphobilinogen, and it is so reported. If the color in the butyl alcohol layer is intense, indicating a heavy concentration of urobilinogen or other Ehrlich-reacting substances, extract the aqueous layer one or more additional times with butyl alcohol to be certain that none of the color in the aqueous layer is due to urobilinogen or other Ehrlich-reacting substances. If there is any doubt as to the identity of the pink or red color in the aqueous layer, it should be examined spectroscopically. Porphobilinogen shows 2 absorption bands, one at 560-570 mμ and a second weaker band at 515-530 mμ.
5. If the aqueous phase is positive for porphobilinogen, prepare a **reagent blank** as follows:
   (a) Measure 5 ml. of a saturated solution of sodium acetate into a No. 6-304 (19 × 150 mm.) Coleman cuvet.
   (b) Add 2.5 ml. Ehrlich reagent and mix.
   (c) Add 2.5 ml. urine. This urine is either undiluted or diluted, depending upon whether dilution was found necessary in step 2 above.
   (d) Add 5 ml. butyl alcohol to the contents. Shake and allow to separate into layers. If the separation on standing is not clear-cut, filter through a moistened coarse filter paper. The butyl alcohol layer comes to the top.
6. With the blank in the cuvet well of the spectrophotometer and at wave length of 565 mμ, set the galvanometer at 100% T.
7. Insert the cuvet containing the test solution and record the % T.
8. From the % T, read the porphobilinogen value (P) from Table 225 (prepared according to the Watson method, in which 2.06 mg. Pontacyl dye mixture is equivalent to 0.6 mg. urobilinogen). In this test, 1 mg. urobilinogen is arbitrarily equated to 1 unit porphobilinogen.

Calculation: Determine the total porphobilinogen content in the 24 hr. urine specimen by substituting the appropriate values in the following formula:

$$\text{Units of porphobilinogen/24 hr.} = P \times \frac{C}{A} \times \frac{V}{100} \times D$$

where

P = units of porphobilinogen in the unknown; obtained by reading the value from the urobilinogen calibration table corresponding to the % T.

C = total vol. diluted aqueous solution in the cuvet. This value is usually 10 ml. (2.5 ml. urine, 2.5 ml. Ehrlich reagent, and 5 ml. sodium acetate).

A = 2.5 ml. This represents the amount of diluted or undiluted urine used.

V = total vol. 24 hr. urine specimen in ml.

D = dilution factor if the urine was diluted. If the urine was not diluted, this value will be 1. If 1 ml. urine was diluted with 4 ml. water, for example, the dilution factor is 5.

Porphobilinogen is not detected in urine of healthy individuals by the methods now available. It is found in large amounts in acute intermittent porphyria (AIP) and to a lesser degree in mixed types of hepatic porphyria. It may be found in small quantities in urine of patients with lead poisoning and in some patients with chronic illnesses such as chronic leukemias and Hodgkin's disease but without symptoms of AIP. Since most patients with these conditions do not excrete porphobilinogen or increased amounts of uroporphyrins, it is believed that the exceptional patient in whom it does occur probably has latent porphyria.

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*Coleman Instruments, Inc., Maywood, Ill.

†Scientific Glass Apparatus Co., Bloomfield, N. J.

Coproporphyrins and uroporphyrins—quantitative assay[42-52]

Principle: Coproporphyrins and uroporphyrins are usually found together in the urine. By acidifying the urine and adding ethyl acetate, the coproporphyrins are extracted with the ethyl acetate, from which they are later extracted by means of 1.5N HCl. The uroporphyrins remain in the aqueous phase and are concentrated by absorbing them onto an aluminum oxide column, from which they are eluted by 1.5N HCl. Since both coproporphyrins and uroporphyrins are fluorescent, their concentrations are measured by reading the fluorescence in a sensitive fluorimeter and comparing the intensity of each with that of a coproporphyrin standard in an identical vehicle. Since uroporphyrin is read against a coproporphyrin standard, a correction factor is necessary in the calculations.

In this procedure 5 ml. urine are used for both coproporphyrins and uroporphyrins. If greater sensitivity is desired when the uroporphyrins are in the normal range, 50 ml. urine may be used to obtain greater fluorescence of uroporphyrins. If 50 ml. are used, a correspondingly larger amount, 400-500 ml., of ethyl acetate must be employed.

Separation:

1. Into a 250 ml. Pyrex separatory funnel, measure the following:
   - (a) 20 ml. distilled water.
   - (b) 5 ml. well-mixed 24 hr. urine specimen.
   - (c) 100 ml. ethyl acetate.
   - (d) 5 ml. buffered acetic acid.
   - (e) 0.1 ml. 1% alcohol-iodine solution. The iodine converts porphyrin precursors to porphyrins.
2. Extract the coproporphyrins into ethyl acetate by shaking the contents of the separatory funnel 30 times, with periodic opening of the stopcock to allow gases to escape. Allow the contents to separate. The aqueous phase, containing the uroporphyrins, goes to the bottom and is drained into a 500 ml. graduate marked **uroporphyrins.** The ethyl acetate phase is retained in the separatory funnel.
3. Wash the ethyl acetate extract containing the coproporphyrins 3-4 times with 20 ml. portions 3% sodium acetate solution until the wash shows no blue-green fluorescence when checked in the photofluorometer. Make the zero setting with sodium acetate. (It is necessary to wash ethyl acetate thoroughly with sodium acetate so that the uroporphyrins which may have been carried over in small droplets of water into the ethyl acetate phase will be carried back into the aqueous portion.) At each washing shake 10 times and allow to separate. The aqueous layer goes to the bottom and is added to the 500 ml. graduate marked **uroporphyrins.**

Coproporphyrins—quantitative assay

1. Extract the coproporphyrins from the ethyl acetate phase in a separatory funnel 3 separate times, using 5 ml. portions 1.5N HCl (approximately 5%). Shake 30 times for each extraction. After each extraction, drain the lower aqueous layer into a graduated cylinder labeled **HCl extract.** Check the third HCl extract in the photofluorometer. Continue the extraction as long as there remains any characteristic orange-pink fluorescence in the last extract. Add all extracts to the HCl extract graduate and make up to a convenient vol. (usually 20-25 ml.), using 1.5N HCl.
2. The **concentration of coproporphyrins** is determined by means of a Coleman photofluorometer (model 12C) using a Coleman No. 12-224 filter as the primary filter. This filter is Corning filter No. 5113, which has its maximum transmission at 405 mμ and which cuts off below 350 mμ and above 480 mμ. A Coleman No. 14-220 red filter is used as the secondary filter. This filter transmits light only above 580 mμ, thus preventing the passage of the incident light and permitting the orange-pink fluorescent rays to pass. These filters are positioned in the photofluorometer. The sensitivity of this photofluorometer is increased up to 15-fold by connecting it to a Coleman spectrophotometer by means of a No. 12-057 connecting cable. Since only the galvanometer of the spectrophotometer is used, the passage of light rays through the spectrophotometer is prevented by turning a spectrophotometer cuvet adapter 90° either clockwise or counterclockwise in the cuvet well. The combination of the 2 instruments just described makes the Coleman photofluorometer a very sensitive instrument.
3. Turn on both instruments and allow them to warm for 10-15 min. The photofluorometer is turned on by means of the Standard control knob which should be turned to its full clockwise position. The neon bulb just above the knob glows when the current is on. Turn the coarse and fine Blank controls of the photofluorometer to full counterclockwise position. Set the aperture control to extreme right.
4. Turn the coarse galvanometer control of the spectrophotometer to the nearly full counter-

clockwise position and the fine galvanometer control to approximately midposition.

5. Place the blank, 1.5N HCl, in a No. 12-190 (105 × 19 mm.) cuvet in the cuvet well of the photofluorometer and cover. Adjust the galvanometer of the spectrophotometer to zero by means of the coarse and fine Blank controls of the photofluorometer. If the galvanometer index does not respond to the zero adjustment, the coarse and fine galvanometer controls of the spectrophotometer are probably at full counterclockwise position.
6. Place the 10 μg coproporphyrin standard in the cuvet well of the photofluorometer. Push the shutter button down, and adjust the aperture control until the photofluorometer meter reads 10. Recheck the zero setting with the blank and then reinsert the standard cuvet and set the galvanometer index of the spectrophotometer at 100 by means of the coarse and fine galvanometer controls of the spectrophotometer.
7. Recheck the zero and 100 settings until the galvanometer response is steady.
8. Insert the unknown solution into the cuvet well, push the shutter button down, and record the galvanometer reading. If the galvanometer reading goes above 100, dilute the unknown quantitatively as necessary, using 1.5N HCl until the galvanometer reading of the unknown is less than 100.

Calculations:

$$10 \times \frac{R}{100} \times \frac{V}{100} \times \frac{100}{A} \times \frac{24\text{ hr. vol.}}{100} \times D =$$

gamma (μg) coproporphyrins/24 hr.
where

10 = μg coproporphyrins used in the standard, equivalent to 10 gamma in 100 ml. 1.5N HCl, the fluorescence is directly proportional to the concentration.

R = reading of unknown.
V = final vol. to which the HCl extract is made.
A = vol. in ml. of urine excreted.
D = dilution factor, if it was found that the unknown had to be diluted to bring the galvanometer reading below 100. If no dilution was necessary, the value of D is 1.

Normal values:
Men: 100-300 μg/24 hr.
Women: 75-275 μg/24 hr.

With moderate to heavy consumption of alcohol these values may reach 350 μg/24 hr. Large quantities of coproporphyrin are excreted in both porphyria erythropoietica and hepatica. In coproporphyrinurias due to lead poisoning, infection, hepatitis, rheumatic fever, etc., there is a moderate to large increase in the 24 hr. output.

Uroporphyrins—quantitative assay

1. To the combined aqueous fractions in the 500 ml. graduate (from step 2 under "separation" described above) add an equal vol. acetone to make a 50% acetone-aqueous mixture.
2. Now set up an aluminum oxide slurry column in a chromatograph tube, as shown in Fig. 340.
   (a) Use chromatograph tube No. C-4080 (size 2, 19 × 200 mm.).
   (b) Introduce a plug of cotton at the upper end of the chromatograph tube. Dampen the cotton with 50% acetone and push it down the tube to plug the openings at the junction. If a chromatograph tube having a sintered glass filter at the bottom is used, the cotton plug may be omitted.
   (c) Make a slurry consisting of Al_2O_2 and 50% acetone containing 1% acetic acid and introduce it into the tube. Not all Al_2O_2 has been found to be satisfactory, and each lot must therefore be checked before it is used routinely.
   (d) Apply suction to the suction flask so that the slurry forms a column in the tube 5-7 cm. high.
   (e) To the top of the column add 2-3 mm. of sea sand which has been washed thoroughly with water and dried. The sand helps to keep the column intact.
3. Add slowly to the tube the 50% acetone solution containing the uroporphyrins and apply gentle suction. At no time during the process of adding the uroporphyrin-containing solution is the solution permitted to drop below the upper level of the slurry column although all the solution except a very small amount on the top of the slurry column is drained through. By means of an ultraviolet light source, it can be seen that the uroporphyrins will concentrate at the upper portion of the column as evidenced by the presence of a concentrated band of orange-pink fluorescence in this region. Wash the column 3 times with 1% acetic acid, each time nearly filling the

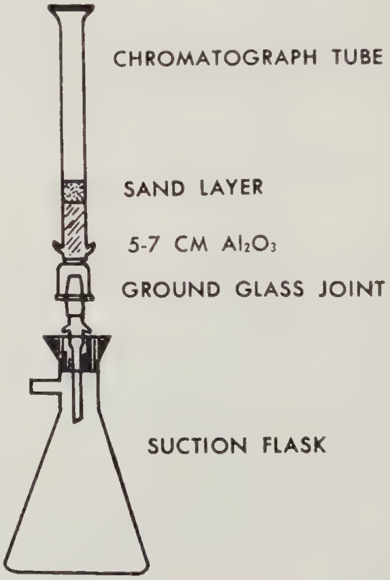

Fig. 340. Chromatographic column for uroporphyrin assay.

Table 225. *Concentration-transmittance calibration table; urine porphobilinogen method (mg.%)*

% T	*0*	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*
10										
20	0.55	0.54	0.52	0.50	0.49	0.48	0.46	0.45	0.44	0.42
30	0.41	0.40	0.39	0.38	0.37	0.36	0.35	0.34	0.33	0.32
40	0.31	0.31	0.30	0.29	0.28	0.27	0.27	0.26	0.25	0.24
50	0.24	0.23	0.22	0.22	0.21	0.21	0.20	0.19	0.19	0.18
60	0.18	0.17	0.16	0.16	0.15	0.15	0.14	0.14	0.13	0.13
70	0.12	0.12	0.11	0.11	0.10	0.10	0.09	0.09	0.08	0.08
80	0.08	0.07	0.07	0.06	0.06	0.05	0.05	0.05	0.04	0.04
90	0.04									

Method factor, 0.897.

chromatograph tube. Check the washings with an ultraviolet light for a blue-green fluorescence of urine and continue to wash until the washings are free of this fluorescence. Check the final washings with a photofluorometer. The washings will normally show the pink-purple fluorescence of acetic acid. Draw off the excess acetic acid in the slurry column with gentle suction, **discard the contents of the suction flask,** and reinsert the stem of the chromatograph tube into the suction flask.

4. Add 5 ml. 1.5N HCl solution to the chromatograph tube and apply gentle suction. By means of the ultraviolet light observe the uroporphyrins (orange-pink fluorescence) as they pass down the column. **Stop** the suction just before the uroporphyrins reach the bottom of the column.
5. Introduce a convenient-sized test tube into the suction flask and insert the stem of the chromatograph tube into the test tube. Add approximately 5 ml. 1.5N HCl to the chromatograph tube, apply suction, and collect all of the HCl extract into the test tube.
6. Remove the chromatograph tube and pour the contents of the collecting test tube into a graduate. Repeat step 5 until the washings no longer show a trace of orange-pink fluorescence when checked in the photofluorometer. All HCl extracts are added to the graduate. Make up to a convenient vol. with 1.5N HCl.
7. To measure the fluorescence intensity of the uroporphyrin extract the same combination of photofluorometer and spectrophotometer is used as described in discussion on coproporphyrins. Set the spectrophotometer galvanometer to zero on the black (lower) scale, using a 1.5N HCl blank and to 100 with a 10µg/100 ml. coproporphyrin standard. If the galvanometer reading goes above 100, dilute the unknown quantitatively as necessary, using 1.5N HCl until the galvanometer reading of the unknown is less than 100.

A coproporphyrin standard is used here because coproporphyrin is much more stable than is uroporphyrin. Since a coproporphyrin standard is used, however, the final results must be multiplied by 0.75, which is the factor required to convert the coproporphyrin reading to the uroporphyrin reading.

Calculations:

Micrograms of uroporphyrins excreted in 24 hr.

$$= 10 \times \frac{R}{100} \times \frac{V}{100} \times \frac{100}{A} \times \frac{24\text{ hr. urine vol.}}{100} \times 0.75 \times D$$

where

10 = coproporphyrin standard of 10 gamma/100 ml. 1.5N HCl.

R = reading of sample.

V = convenient vol. to which HCl extract was diluted in step 6.

A = vol. urine used. In most cases this will be 5 ml., but occasionally larger amounts will be used.

0.75 = transmission conversion factor to convert coproporphyrin reading to uroporphyrin reading

D = multiplication factor if a dilution was made in step 7.

Normal: 10-40 µg/24 hr.

WHITE BLOOD CELLS OR LEUKOCYTES

Although the level of erythrocytes is fairly constant in health, the level of leukocytes is subject to so many variations that it is difficult even to determine a normal level. The normal value is not influenced by sex or race.

The average total leukocyte count of the adult is 7400/c.mm. with a range of 4500-11,000/c.mm. Variations occur outside this range, an excess beyond 11,000 being called leukocytosis and a decrease below 4500 leukopenia. The average distribution is 40-75% granulocytes and 15-45% lymphocytes. Variations may occur in the blood of the same individual, even in health, from day to day or at different hours of the same day. Some authorities have spoken of digestive leukocytosis, supposedly associated with food intake. There is some question as to whether there is justification for such a designation since variations may occur quite independently of meals.

Medlar[53] investigated the differences between leukocyte counts in individuals at rest and after exercise and found that there is no such thing as an absolutely constant leukocyte level and that fluctuations in the leukocyte picture are very inconsistent. A fluctuation of more than 50% of the total count and of over 8% in the differential count must occur before much significance can be placed on the changes in these values.

Apart from the diurnal variation, there are variations as a result of age. At birth, the leukocyte count averages 18,100/c.mm., with a range of 9000-30,000. At the end of 1 week, there is a drop in the total leukocyte count to an average of 12,200, with a range of 5000-21,000. These levels tend to persist until the end of the first or second year and then continue to fall within the normal average. Until the age of 4 to 5 yr. has been reached, there is a tendency toward the slightly higher leukocyte counts within the normal adult range.

The leukocytosis of the first few days of life is mainly due to polymorphonuclear cells which then begin to fall, whereas the lymphocytes begin to increase. At the age of 4 to 5 yr. the lymphocytes begin to fall again, and the granulocytes increase.

The white count does not seem to be affected appreciably by altitude.

During pregnancy, there is usually an elevated count of approximately 10,000-10,600, or even as high as 15,000. By the fifth to ninth week post partum, there is a return of the total leukocyte count to within the normal average and normal range.

Physical exertion produces leukocytosis. Stress and emotional distress of long duration are followed by an elevation of leukocytes and an increase in lymphocytes.

FATE AND PHYSIOLOGY OF WHITE BLOOD CELLS

At this time neither the duration of life nor the mode of destruction of the white cells is definitely known. It is generally believed that disintegration of leukocytes in the blood stream does occur. It is probable that white cells are consumed in greater amounts than the red cells. Since there are only $\frac{1}{1000}$ as many white cells in the peripheral blood as red cells, the time of their development must be longer or their life must be much shorter. There must be, according to the present figures, at least an age of 2 hr. for a leukocyte. Ponder, on the basis of Arneth's shift to the left, attempted to calculate the life of leukocytes and found it to be from 2-3 weeks. The segmentation of white cells according to Schilling[54] is not a measure of their age. Magner[55] states that the life of the leukocytes is probably not longer than a few days, but that some workers believe that the nonmotile cells which are seen in supravitally stained preparations are artifacts. Many leukocytes penetrate the capillary walls, wander through the tissues, or escape from the body through mucous surfaces. By virtue of their phagocytic powers and by the production of bacteriolytic substances, they are active in the defense of the body against pyogenic bacteria, and they also play an important part in the digestion of necrotic tissues by means of proteolytic enzymes.

Leukocytic life is rhythmically regulated. The contribution of Sabin et al.[56] covers the question of the normal rhythm of the white blood cells. This contribution has given us a better understanding of the physiology of the white blood cells. The study was worked out through supravital technic. As described by Sabin,[57] a type of cell exists in the blood which she called the nonmotile leukocyte. This loss of movement is essentially a chemical and physical change in the neutrophilic granules, together with a loss of power to react to vital stains. This cell was first described by Schilling[58] in 1908. He noted the cessation of motility and also described the subsequent disappearance of the centrosphere. He maintained at that time that it represented the physiologic death of the leukocyte. Cunningham, Sabin, and Doan noted the showerlike appearance of these dying cells in the blood. They believed that if one could arrive at an estimate of the amount of cell death of the leukocytes in the blood stream one might devise some method of analyzing the rhythm of the leukocytes. Counts of the white cells of human subjects at different times of the day had previously been made. Torday[59] found the lowest count in 61 patients was 3130 and the highest 9800. Turk[60] found the normal count at different times of day was from 5000-10,000 white cells. Most of the studies on the rise and fall of leuko-

Table 226. *Staining characteristics (Wright-Giemsa) of cells of granulocytic series*

Nucleus	*Cytoplasm*
Myeloblast (Naegeli): round, finely reticular in structure, with blue plastin nucleoli	Highly basophilic, no granules; faint to strongly positive oxydase reaction
Promyelocyte (Pappenheim): round, with a tendency to polymorphism and lobulation, especially true under pathologic conditions; plastin nucleoli at times faint or completely absent; whole structure denser and darker	Dark blue to pale blue; beginning deep red azurophilic finer or coarser granules (progranules)
Myelocyte (Ehrlich): round, oval, to slightly indented, only chromatic nucleoli; coarser and darker interstices	Pale blue to neutral, mature neutrophilic, basophilic, or eosinophilic granules
Juvenile granulocyte (Schilling): sausage-shaped, well-filled structure distinctly divided into fields, frequently with polar nucleoli	Occasionally slightly basophilic, usually neutrophilic granules very intensely stained, with a tendency to azurophilic characteristics
Stab or *rod nuclear granulocyte* (Schilling): slender, darker, often with a twisted nucleus with bizarre indentations suggestive of beginning segmentation	Mature granules
Segmented granulocyte (Schilling): nucleus with many divisions; 2-5 segments connected in a chain by fine threads or bridges	Mature granules

cytes have been made in pathologic conditions.

Investigators have found that the injection of foreign proteins, bacteria, drugs, etc., produces very definite leukopenia followed by leukocytosis. It has been found by some observers that variations in the blood pressure are followed by changes in the number of blood cells, a lowering in the pressure being followed by a decrease in the number of cells. Arneth[61] and other investigators have found that there is a definite increase in the number of white blood cells after a cold bath. Change in posture has been followed by variation in the number of leukocytes. It was also found that muscular work caused a constant increase of leukocytes and that this disappeared in the course of 2-3 min. but reappeared again on renewed exercise.

Digestive leukocytosis has been studied by a number of investigators. Pohl[62] studied digestive leukocytosis in dogs after 18 hr. of fasting and found after feeding them meat that they had leukocytosis beginning in 1 hr. and reaching a maximum at 3 hr. With nonalbuminous foods he obtained no leukocytosis. Rieder[63] confirmed Pohl's findings both in man and in dogs. Sirensklj,[64] in studies on man, found digestive leukocytosis after meals rich in albumin or in fat but only a very slight reaction after food consisting of carbohydrates alone. Pirone[65] from histologic studies of the blood-forming organs during digestion concluded that the reaction had 3 phases: (1) increased destruction of the older blood cells, (2) increased stimulation to the formation of new cells and the migration of elements into the blood stream, and (3) signs of hyperfunction and increased hematopoietic activity in the bone marrow, spleen, and lymph glands. The work of Cunningham and associates has given the best information on the question of the rhythm of white cells. It is fairly constant for 7-8 hr. as shown by supravital preparations. They showed that the white blood counts are higher in the afternoon than in the morning quite independently of whether food is taken.

Physiology of polymorphonuclear leukocytes

The physiology of the formation of granulocytes has been thoroughly studied. We know that granulocytes, like erythroblasts, are formed in well-defined centers and that promyelocytes and myelocytes, characterized by blue cytoplasm and light, nucleolated nuclei, develop from these centers. In the bone marrow before this stage has arrived in the maturation of granulocytes, there can be seen large basophilic cells with nucleolated nuclei, the so-called Pappenheim's lymphoidocytes, Maximow's polyblasts or primitive cells, Ferrata's hemocytoblasts, or Schilling's undifferentiated parent cells. Schilling[54] illustrates the various stages through which the granulocyte passes before it becomes a fully mature segmented granulocyte.*

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*See Table 46 in Schilling, V.: Handbuch der normalen und pathologischen Physiologie, part 2, Berlin, 1928, Julius Springer, p. 789.

In this connection attention must be called to the differences between the Arneth and Schilling theories of shifts in blood pictures, which is discussed under remarks on differential counts.

The physiologic importance of the segmented forms lies in their phagocytic activity, their enzyme production, and their ameboid motility.

Basophilic granulocytes: Basophilic granulocytes are the blood mast cells and are related to the tissue mast cells which are occasionally encountered in bone marrow smears. These cells produce and store heparin and are related to the production of hyaluronic acid and serotonin. See discussion of mast cells.

Eosinophilic granulocytes: Barnhart and Riddle,[66] in Seeger's coagulation laboratory, have shown by immunologic methods that eosinophils are the only cellular source of plasminogen (prefibrinolysin). Eosinophils are commonly seen in immune or allergic reactions and are dependent on hormonal activity.

Physiology of lymphocytes[67-73]

According to Sabin,[67] lymph sacs are formed in the embryo of 6-24 mm. near the jugular vein. These sacs become the so-called lymph hearts. The latter form the primary lymph glands (embryo from 10.5-30 mm.). The first lymph follicles appear in the embryo of 50 mm. Lymphocytic cells develop from the mesenchymatous migratory cells of the adventitia of blood vessels in the tissues between the lymphatic capillaries. Maximow[68, 70] designated the first free mesenchymatous migratory cells as lymphocytes. He insisted that from the primitive cells true small lymphocytes may form and that even the lymphocyte of the blood preserves this polyblastic embryonal capacity. There is good evidence that in a 4 mo. fetus the lymphocytes originate in the thymus. Later they are produced in the spleen and lymph nodes. The first circulating lymphocytes are of the large type, whereas in the second half of gestation the small lymphocytes take over and form the majority of the circulating white cells.

The follicle is the unit of true lymphatic tissue. Lymphatic tissue is found extensively all over the body. The lymph nodes and spleen furnish the true lymphocytes to the blood stream. The follicle has a germinating center. This consists of lighter and larger cells with vesicular nuclei and distinct nucleoli.

Flemming believed that the formation of lymphocytes emanates from the cells of the germinating centers. Naegeli and Schridde call these cells lymphoblasts. These lymphoblasts are offspring of reticulum cells and give rise to large lymphocytes which in turn change to small lymphocytes. This transition from large lymphocytes to small ones has been proved by incorporating radioactive tritium-labeled thymidine into the deoxyribonucleic acid of the cells in the stage of DNA synthesis (mitosis). The life span of lymphocytes labeled with P^{32} or chromium^{59} is about 100 days. They circulate from the blood to the lymph nodes and return by means of the efferent lymphatics and the thoracic duct. Lymphocytes are destroyed in all the organs in which they are formed (liver, spleen, thymus, intestines, lung, and lymph nodes). The production of lymphocytes is influenced by adrenal cortical extract which, when administered in excess, diminishes the circulating lymphocytes to 50% or more of the original level.

The lymphocyte is probably not a homogenous cell type. In certain conditions lymphocytes can give rise to plasma cells, as seen in macroglobulinemia, in which one observes transitional stages between lymphocytes and plasma cells in the bone marrow. By electron microscopy, lymphocytes never show an ergastoplasm, which is characteristic for the globulin-producing plasma cells. In this connection the condition of alymphocytosis described by Hitzig[73] is of importance since it is characterized by absence of lymphocytes, plasma cells, and gamma globulin. The protective effect of administration of splenic emulsion or of shielding the spleen during total body irradiation in experimental animals has brought up the problem of conversion of lymphocytes to pluripotential reticulum cells from which marrow regeneration can occur. The pathology and the interrelationship of the various types of lymphomata also suggests such a conversion possibility.

Schilling observed true lymphocytic cells in bone marrow, especially in chronic conditions. They may form lymph follicles. There is no evidence that their presence foreshadows the future development of one form or another of lymphoma.

We know that in the adult about one fourth of the circulating white cells are lymphocytes, but they are only a small part of the total number manufactured. Young immature lymphocytes, not blast cells, may occur in the peripheral blood in apparently normal persons and occur frequently in lymphocytic leukemoid reactions, such as virus diseases, hepatitis, and infectious mononucleosis (virocytes). In contradistinction to the young lymphocytes, the more mature lymphocytes are present in the intestinal tract and lymphatic system rather than in the blood stream.

The **function of lymphocytes** is recognized in the walling off of chronic inflammatory foci. They are increased in chronic inflammatory states. They are increased also during infancy and in recovery from acute infections and in certain diseases such as measles, mumps, whooping cough, and undulant fever.

Lymphocytes are slowly moving cells which under certain circumstances show phagocytic activity and are able to leave the confines of the blood vessels to become tissue cells. They may transform into large ameboid macrophages. Some lymphocytes appear to be responsible for antibody formation and may during antibody synthesis transform into plasma cells. One of the most interesting aspects is their relationship to the delayed type of allergy of skin reactions (e.g., tuberculin test). Apparently this response is linked to the lymphocyte and is transferable from patient to patient by lymphocytes. It is independent of serum globulin levels or of other humeral factors.

Physiology of monocytes

Ehrlich called all cells with eccentric pale nuclei and large faintly basophilic cytoplasm, generally without granulations, large mononuclear cells. He differentiated these from transitional cells which had deeply indented nuclei, constituting the beginning of neutrophilic granulations. He believed that these cells were transitions to neutrophils. Pappenheim claimed that they were older lymphatic forms of the Rieder type. All authors except Pappenheim traced these cells to the neutrophilic system. Einhorn and Ehrlich believed that a relationship existed between these cells and the spleen and called them splenocytes. Pappenheim and Ferrata in 1910 finally suggested the name monocytes for both the mononuclear and transitional cells. Pappenheim[74] was the first to identify the monocytic granules as **not** neutrophilic and **not** true granules, and Naegeli admitted in the same year that the granules were azurophilic and not neutrophilic. Schilling emphasized that the oxydase reaction indicates that the monocytes occupy something like an intermediate position between the other systems. He demonstrated basophilic juvenile forms in variola and also the close relationship between the blood monocytes and endocarditis lenta. He concluded that the peripheral macrophage is merely a functional form, coming from the same parent cells as the monocytes, and that the monocytes could be converted into macrophages.

Schilling sought to find the genesis of monocytes in the reticuloendothelium structure, that is, the endothelium of hematopoietic organs and the Kupffer cells of the liver. Maximow opposed this view with his histiocytic or lymphocytic origin of monocytes. He explained the monocytes as physiologic blood polyblasts.

The functions of monocytes are phagocytosis and storage. Phagocytosis is least developed in the bone marrow monocyte and best developed in the tissue monocyte. The latter may be stationary or mobile. They respond to adrenal cortical extract in the same way as lymphocytes; they decrease 50% or more.

Plasma cells

Plasma cells are closely related to lymphocytes and reticulum cells. In macroglobulinemia, various transitional forms can be seen which have been named lymphoid reticulum cells and lymphoid plasma cells or lymphocytoid cells. The normal bone marrow plasma cell produces gamma and beta globulins or is at least responsible for their release. Under pathologic conditions, the plasma cell is responsible for the modification of plasma proteins and the production of paraproteins, macroproteins, and various abnormal protein states (dysproteinemias), e.g., cryoglobulins. As antibodies consist mainly of gamma globulins (but not all gamma globulins take part in the antibody formation), the plasma cells are closely related to the resistance of the organism. The rare plasma cell appearing in peripheral blood is not involved in protein production. Various morphologic findings such as intercytoplasmic Russell bodies and crystalline inclusions, as well as clinicopathologic findings, speak for the role of the plasma cell in protein synthesis. In pathologic conditions associated with hypergammaglobulinemia there is usually an increase in bone marrow plasma cells. Hypergammaglobulinemia or hyperbetaglobulinemia is usually associated with multiple myeloma. The role of the plasma cell in protein metabolism is challenged by such conditions as mutliple myeloma without hypergammaglobulinemia and macroglobulinemia and amyloidosis without an increase in plasma cells. In agammaglobulinemia and hypogammaglobulinemia there may be lymphoid hypoplasia or hyperplasia, but there is usually a disturbance in production of plasma cells and thus a diminution in bone marrow plasma cells. The ergastoplasm within the cytoplasm is responsible for the protein production, as shown by electron microscopy.

BLOOD PLATELETS

Blood platelets appear in the stained specimen as single, bright, azure, rounded, or elongated small bodies with a delicately striped and granular structure. They are about ½ to ⅕ the size of red cells.

The past dispute as to the source of blood platelets is of historic interest only. Bessis has shown in beautiful phase contrast pictures the formation of platelets within the megakaryocytes and their release into the bone marrow. Kinosita in a motion picture showed the production of platelets by megakaryocytes observed through windows in rabbit bones. Vazquez by means of fluorescent antihuman platelet serum proved the presence of at least one common antigen in platelets and megakaryocytes. Earlier histochemical studies of acetylcholinesterase led to similar conclusions.

Each platelet consists of a central body, a granulomere, and a surrounding hyalomere which forms pseudopodia.

Chemically the platelets are rich in adenosine triphosphate (ATP) and glycolytic enzymes. The ATP content is apparently related to clot retraction. Platelet phospholipids (phosphatidylserine) increase blood coagulability in vitro. Phosphatidyl ethanolamine is active in the coagulation mechanism only when combined with lecithin. As already stated, in the embryo platelets make their appearance at the same time as megakaryocytes can be found. The platelets are produced by the megakaryocytes in bone marrow. Megakaryocytes are also found in the lung and spleen but are apparently not platelet-producing in these tissues. Platelets survive 3-6 days in the peripheral circulation and are then destroyed in the spleen.

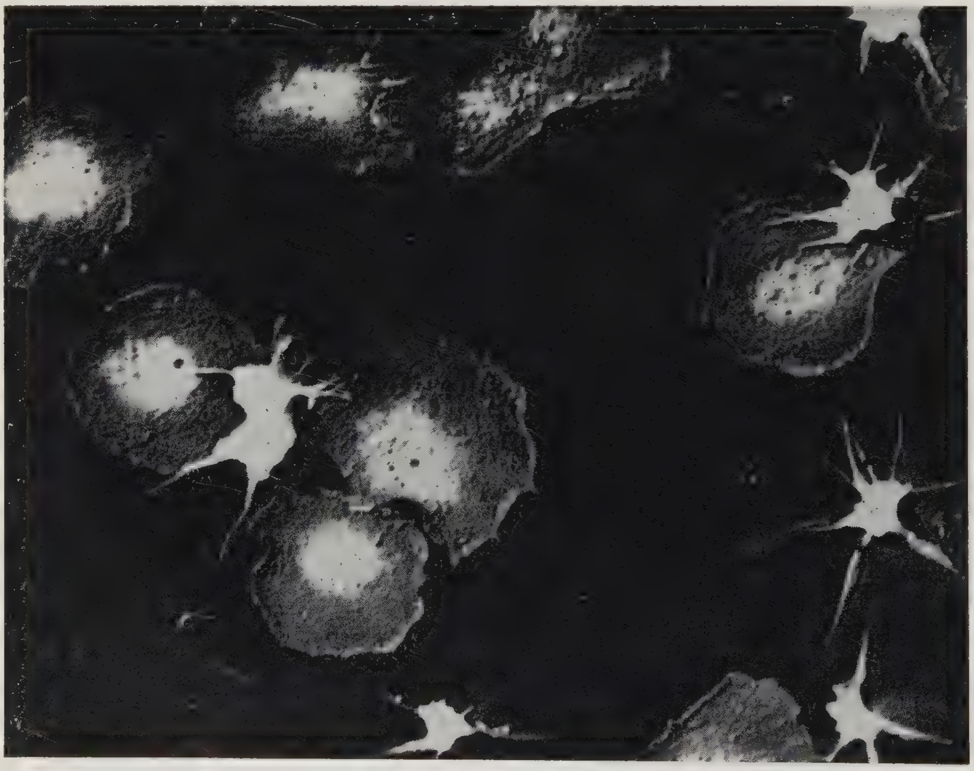

Fig. 341. Normal platelets with pseudopods and spreading of hyaloplasm. Electron micrograph. (Courtesy Prof. H. Braunsteiner, Vienna, Austria.)

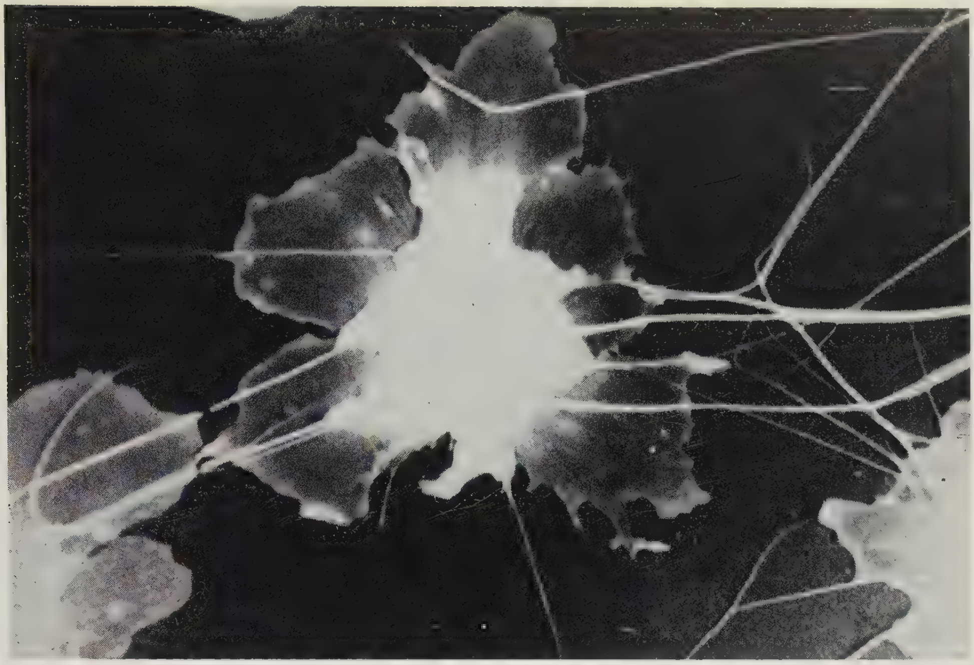

Fig. 342. Platelets in early stages of coagulation, showing viscous metamorphosis. (Courtesy Prof. H. Braunsteiner, Vienna, Austria.)

The total number of circulating platelets is fairly constant in health and is not subject to great variations. In normal plasma there exists a thrombopoietic principle which stimulates megakaryocytic maturation and platelet production.[75]

Functions of platelets[77, 78]

The functions of platelets are concerned with hemostasis and blood coagulation and can be broken down into several phases which in vivo may well occur at the same time.

Initiation of blood coagulation: By means of their adhesiveness, platelets adhere to the injured intima of the blood vessel and release platelet factors which lead to platelet agglutination and formation of platelet plugs. Electrostatic forces have been suggested as the basic mechanism of platelet adhesiveness. On the other hand, deposition of minimal amounts of fibrin may be the initial mechanism.

Vasoconstriction: When platelets disintegrate, they release 5-hydroxytryptamine, a substance which is formed in the gastrointestinal tract by argentophil cells and is transported and concentrated by the platelets; 5-hydroxytryptamine is called **serotonin,** and probably in unison with a second platelet factor it acts on the arterioles, leading to narrowing of the lumina. Thrombin may be required for the release of 5-hydroxytryptamine which interacts with a plasma factor. The metabolism of serotonin results in the production of 5-hydroxyindoleacetic acid which is excreted in the urine and can be detected by appropriate tests.

Platelet agglutination: The agglutination of platelets concerned with hemostasis differs from the immunohematologic platelet agglutination. Normal platelet agglutination can be produced by many substances such as peptone, heavy metals, plasma, serum, saponin, etc. Heparin prevents platelet agglutination.

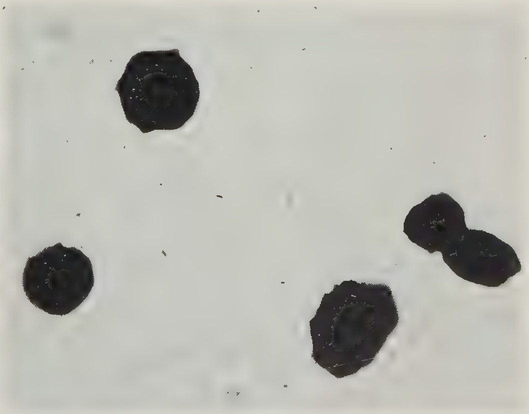

Fig. 343. Platelets in thrombocytasthenia. Defective pseudopod formation and lack of spreading. (Courtesy Prof. H. Braunsteiner, Vienna, Austria.)

Immune agglutination is considered in the discussion of immune thrombocytopenia.

Platelet groups and types: Platelets belong to the A-B-O system and to several subgroups. This aspect is considered in the discussion of immune thrombocytopenia.

Platelet factors: According to Seeger,[76] platelets contain 11 factors such as prothrombin activator, thrombin activator, accelerator globulin-like factor, antiheparin factor, etc.

Clot retraction: Clot retraction is a characteristic function of platelets producing contraction of the fibrin network of the fresh clot. Viscous metamorphosis of the platelets precedes their action on the fibrin network. Clot retraction parallels platelet adhesiveness and aggregation, as well as being related to the number of platelets. Viscous metamorphosis of the platelets first leads to platelet aggregation, then to platelet fusion, and finally to release of platelet factors. Some of these aspects are considered again in the discussion of coagulation.

Physiologic variations of the platelet count

Normal values (indirect method): Normal values for the indirect method are 250,000-400,000/c.mm. blood. Various counting methods give different normal values for platelets. The indirect method, which determines the number of platelets per 1000 red blood cells, is influenced by the distribution of platelets on the slide, whereas the direct method of counting platelets is influenced by the breakdown of platelets in the counting fluid.

Variations in the number of platelets occur from individual to individual but are only slight in a given individual during each 24 hr. period. An increase in the number of platelets is noted from birth to the age of 3-12 mo., after which normal values are obtained.

The behavior of platelets in conditions accompanied by or leading to venous thrombosis has been extensively studied. Two such conditions are pregnancy and surgical operation. During pregnancy the platelets are normal, but after delivery their number increases for about 7-10 days. Similar platelet increases may be seen following surgical operation. Splenectomy is characteristically followed by a temporary (seldom permanent) increase in platelets so that some splenic controlling mechanism can be postulated.

Chapter 61

Technics in hematology

JOHN D. BAUER

INTRODUCTION

Blood examinations are important in completing the examination of every patient. These tests must be performed with consummate technical skill, utilizing procedures and equipment best suited for various situations. Certain hematologic tests can be considered as basic routine "screening" procedures. These should include hematocrit and hemoglobin determinations and a white blood cell count, with examination of a properly prepared blood spread. The results of these tests will often point the way to other special procedures, or the history and physical examination may indicate the need for more exhaustive hematologic investigation of the patient. In the ordering of tests or the interpreting of results, knowledge of the normal ranges, rather than some fixed number, and of the limitations of accuracy of the procedures are of inestimable value to the physician.

Sound planning in the laboratory implies a thorough knowledge of equipment and procedures which can be gained only by consistently keeping abreast of such matters by reading the literature and by attendance at meetings especially aimed in these directions. The purchase of equipment should be based on the work to be done, as well as on the accuracy of the instrument. For example, photoelectric hemoglobin methods lend themselves readily to assembly line organization, which makes them a real economy in technician time. At the same time, maximum accuracy can be obtained by use of a photometer. In a laboratory doing a large volume of work, such equipment becomes almost a necessity. However, the less expensive visual hemoglobinometer may serve satisfactorily in a physician's private office if the volume of work is minimal. In either case, knowledge of the limitations of the equipment is necessary for proper appreciation of the results. An improperly standardized expensive photoelectric apparatus may be more inaccurate than less expensive equipment. In both instances, careless technic will militate against accuracy.

The choice of procedures and full knowledge of their limitations should be considered. Frequently, relatively simple procedures will suffice and thus eliminate more complicated and more time-consuming technics. The estimation of blood platelets is a good example of such a situation. In a properly prepared blood spread, if platelets are seen in every oil immersion field, it can be safely said that thrombocytopenia does not exist. In clinical conditions with bleeding diathesis and obvious normal numbers of platelets in the spreads, the actual counting of platelets is unnecessarily time-consuming. On the other hand, if trends in numbers of platelets are to be determined, then platelet counts by more precise methods are in order. There may be situations in which simple or commonly performed tests may not reveal any abnormality and it might be necessary to perform some little known or infrequently used test to uncover the basic disorder. An example of such is the plasma thromboplastin component (factor IX) determination in a patient with a bleeding diathesis with normal routine test results.

These are but a few examples of intelligent choice of procedure indicated by the clinical state of the patient. The choice of technic may be influenced by the range of accuracy desired or desirable. Erythrocyte counting, for example, lends itself to scrutiny from this standpoint. The primary purpose of a red blood cell count is determination of the presence or absence of anemia or polycythemia. In routine work, significant deviations can be determined simply with an accuracy of ±5% for hemoglobin and ±2% for hematocrit. On the other hand, routine erythrocyte counts using 1 pipet, 1 chamber, and 500 red blood cells are subject to an inherent error of ±16%.[79, 80] This means that a red blood count that is actually 5,000,000/c.mm. may be reported as 4,200,000 or 5,800,000/c.mm. at either extreme. These discrepancies in the limits of accuracy of this test should make it quite obvious as to the choice of tests for routine work, namely, the elimination of the red blood count. If the hematocrit and hemoglobin tests reveal subnormal values, then a well-made erythrocyte count is indicated. We heartily endorse Ham's[80] recommendation as to the minimum procedures for red blood cell counting for clinical use: 1 pipet, 2 chambers in the hemacytometer, and counting 400-600 cells per chamber. For determination of the corpuscular constants (mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration), Ham recommends using 2 pipets and 2 chambers and counting 400-600 red cells in each chamber.

It is of utmost importance that technicians present an appearance to the patient and to the physician that reflects confidence. Laboratory technicians should appear neat and clean; particularly is this true of the hands. Constant observation, reading, and the repeated performance of tests will aid in bestowing confidence in oneself, which will be reflected in the patient's response. It is important for technicians to become thoroughly familiar with all phases of every test that they perform and to know the limitations of the particular test that they are reporting. There is too great a tendency for some persons to believe that results obtained in many tests are rigidly accurate scientific figures. For this reason, in the following section on technic and in some of the preceding sections, a mean average and a range of the test are included. The standard values that follow have largely been taken from *The handbook of biological data.*[81] This compendium of data was obtained by contributions "by experts in the various areas of hematology. These tables have been reviewed by outstanding specialists in the subjects covered and represent an unusually complete collection of data on blood, which is unique in the high degree of reliability. Over 600 leading investigators in biology and clinical science were consulted for the accumulation of these data. In addition, numerous outstanding experts reviewed the material prior to publication for its accuracy."*

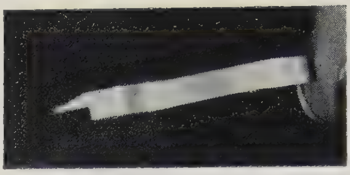

Fig. 344. Hemolet. (Courtesy Scientific Products Division, American Hospital Supply Corp., Evanston, Ill.)

The one procedure common to all hematologic tests is the drawing of blood samples from patients. This procedure must at all times be carried out with full knowledge and appreciation of asepsis. **All materials which touch the patient for obtaining a blood specimen must be sterile.** This means sterile needles, cotton, and syringes. The skin must be thoroughly cleansed with some antiseptic—alcohol, alcohol-ether mixture, acetone, Merthiolate, Zephiran, or other preparations. After a skin area has been cleansed, the operator must not touch the area with the fingers. It is too common a practice to locate a vein in an arm, cleanse the area, and then proceed to palpate the area again with the nonsterilized finger before inserting the needle. **It must be repeated that if the finger touches the area to be punctured, this area must be cleansed again.**

Instruments used to puncture the skin: Any instrument used to puncture the patient's skin for the purpose of obtaining blood for examination must be absolutely sterile. **This does not mean that one may use a needle attached to a cork which is carried point downward into a small bottle containing alcohol or any other chemical or disinfecting liquid.** Sterility means just what it implies, namely, actual killing of bacteria or viruses. It is well known that cases of infectious hepatitis, for instance, have occurred in hospitals when imperfectly sterilized needles and lancets have been used.

Four different methods are suggested. (1) Break sharp-pointed pens in half, wrap a number in paper, and sterilize by dry heat at

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*From Albritton, E. C. (editor): Handbook of biological data: Standard values in blood, Philadelphia, 1952, W. B. Saunders Co.

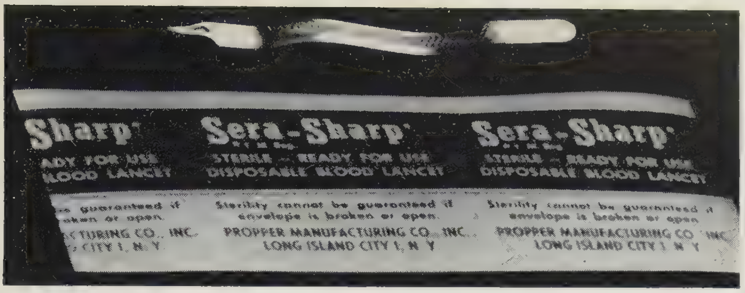
Sharp
Sera-Sharp
Sera-Sharp
STERILE — READY FOR USE
DISPOSABLE BLOOD LANCET
Sterility cannot be guaranteed if envelope is broken or open.
PROPPER MANUFACTURING CO., INC.
LONG ISLAND CITY 1, N. Y.

Fig. 345. Sera-Sharp. (Courtesy Propper Manufacturing Co., Inc., Long Island City, N. Y.)

170° C. for 1½-2 hr. (2) Use Hemolets* (Fig. 344). Hemolets are razor-sharp ribbons of stainless steel packaged in sterile wrappers. They are thrown away after being used once. (3) Use Sera-Sharp† sterile disposable lancets (Fig. 345). These also are used once and discarded. They are inexpensive and need not be used more than once. When Hemolets or Sera-Sharp lancets are used, the possibility of transmitting infectious diseases such as virus hepatitis, homologous serum jaundice, malaria, etc. through skin puncture can be precluded. Both the Hemolet and the Sera-Sharp make the puncture about the correct depth, not too shallow and not too deep. (4) Use the Steri-Lance† (Fig. 346).

To use the Steri-Lance, place all lancet heads into the side of the container marked Sterile. Place the lancet holder in the center groove. Sterilize the complete unit at 15 lb. pressure for 30 min., in an autoclave.

To attach the blade unit, slip the end of the holder over a sterile lancet head and screw on securely with a few turns of the holder. Holding the assembled lancet with the blade up, turn the screw cap of the opposite end until the tip of the blade is just barely visible. Once adjusted, the holder will give a uniform depth of penetration with all blades. The lancet blade is not attached to the spring but has a special recoil action. Pull out the top cap of the holder until the spring catches, place the tip of the lancet blade against the disinfected skin in the usual manner, and press the release level.

After use, wipe the blade clean, then place the lancet head in a pocket on the unmarked side of the insert, and unscrew by twisting the handle. **It is never necessary to touch the lancet blade or head at any time.** After all the blade units have been used, the complete unit is ready for sterilization. To prevent confusion, do not transfer blade units from unsterile to sterile side until they are ready for sterilization.

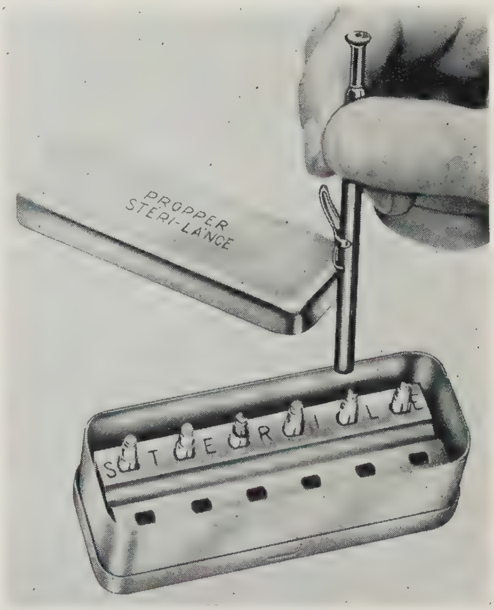

Fig. 346. Steri-Lance, an adjustable automatic lancet for skin puncture. (Courtesy Propper Manufacturing Co., Inc., Long Island City, N. Y.)

Technic of puncture:

1. To use the sterile pen point, Hemolet, Sera-Sharp, or Steri-Lance, hold the lobe of the ear between the thumb and forefinger.
2. Place the puncture point on the lobe of the ear and make a quick incision. If the hand is used, hold the hand tight before making a jabbing puncture. Blood should ooze in small drops spontaneously or with very slight pressure. **Do not use strong pressure to increase bleeding.** Such pressure causes the blood films to become contaminated with tissue juice and endothelial elements and alters the patient's counts.
3. Remove the first small drop of blood and then proceed with the various counts and blood spreads. When finished, wipe the skin with alcohol and discard the used cotton.

Proper types of blood samples are important; their source is often dependent upon the nature of the test to be performed. Some routine hematologic procedures such as red blood count, white blood count, hemoglobin, and blood spread can be obtained from capillary blood taken by simple skin puncture, as directed above.

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*Scientific Products Division, American Hospital Supply Corp., Chicago, Ill.

†Propper Manufacturing Co., Inc., Long Island City, N. Y.

Blood specimens for many hematologic procedures are obtained in many hospitals by venipuncture.

To use venous blood: The method of making a venipuncture in an adult is given on p. 29.

Venous blood must be transferred quickly to a suitable anticoagulant to prevent clotting. A satisfactory anticoagulant mixture known as **3:2 oxalate mixture** is prepared as follows:

Ammonium oxalate . . .	1.2 gm.
Potassium oxalate	0.8 gm.
Distilled water	100 ml.

1. Use 0.1 ml. for each ml. blood after the anticoagulant has been dried. For routine work requiring 5 ml. blood, place 0.5 ml. into test tubes or suitable wide-mouthed bottles.
2. Allow to dry or heat gently in an oven to evaporate the water, which leaves a white dry powder on the bottom of the collecting vessels. Stopper and keep away from dust.
3. Place the blood into the anticoagulant tube and invert gently 5-10 or 20 times to ensure adequate mixing of the anticoagulant and blood.

Venipuncture in infants: To obtain a large sample of venous blood from an infant one may perform a femoral or jugular vein puncture. Both procedures are relatively formidable and should be undertaken only by a physician and not by a technician.

For small quantities of blood one can easily obtain samples by puncture of the baby's thumb, large toe, or heel. This should be done under strict asepsis, namely, cleansing of the skin and of the puncture points with an antiseptic soap and water or by saline solution and then using one of the skin antiseptics, such as a 1:1000 solution of tincture of Merthiolate. After this is applied and allowed to dry, punctures are made with an extremely sharp-pointed surgical knife. One with a long, tapered point is preferred (Bard-Parker blade No. 11). When several of these punctures are made, with constricture of the ankle, enough blood can be obtained to carry out a serologic procedure and chemistry determinations if the heel or toe is used.

Blood kept in the anticoagulant may be used for making erythrocyte counts, hemoglobin determinations, white blood counts, sedimentation rates, hematocrit determinations, fragility tests, reticulocyte counts, icterus index, and other tests, if performed within 3-4 hr. after collection of the blood sample.

Blood taken in an anticoagulant is generally unsatisfactory for the preparation of blood films because both the leukocytes and the red blood cells show morphologic alterations that render cell identification difficult at times. With a little practice, it is possible to take a small drop of blood from the needle at the time of venipuncture, place it on a glass slide, and make a satisfactory blood spread.

Precautions to be observed in handling blood for hematologic study: The blood sample should be kept away from excessive heat, such as a radiator under a laboratory table or an oven, since heat will hemolyze the blood.

Thorough mixing of the blood specimen prior to removal of samples for each examination is necessary to secure true sampling of the specimen.

One advantage of collecting blood routinely in an anticoagulant is that tests may be repeated and extra procedures can be carried out if indicated without recalling the patient and repeating the venipuncture. Another advantage is the multiple number of tests which may be performed without concern about clotting of blood before pipetting has been done or closing of the puncture wound before sufficient specimen has been collected.

Glassware must be thoroughly cleaned and dried. Immediately after they are used, the pipets, counting chambers, hematocrit tubes, etc., must be cleansed with cold water to prevent the glassware from being stained with dried blood. If these instruments become brown, they must be treated with strong acids or ammonium hydroxide, which is undesirable.

Slides and cover slips used for preparing blood spreads must be new and not reused, and they should be cleaned thoroughly. Ordinary glass slides may be cleaned readily by the vigorous use of clean dry gauze. The fingers must not touch the edges of the slide nor the slide surfaces.

The care and use of the microscope are discussed in Chapter 1. For oil immersion work, maximum light intensity with the condenser in the closest position to the blood film and the light shutter open to its maximum will give the clearest picture of the fields.

Reporting results of laboratory tests is an important function of the laboratory. Even the most meticulous technic requiring many hours of time would be worthless if the results were not recorded in some proper manner. Reports should display normal values and ranges for normal values, as well as the result of a particular test. Duplicates of reports should be retained by the laboratory for future reference. Promptness in reporting will gain the plaudits of the referring physician. Reports on examination of blood films should be so worded that the referring physician can visualize the film. Laboratory reports constitute one link in the chain of confidential relationship between patient and physician. Laboratory technicians have been taken into this confidence. **Under no circumstances should they betray this confidence by revealing reports to persons unauthorized to receive such information.**

All material, such as blood specimens and reagents, must be properly labeled in order to avoid both confusion and error. Labeling of blood should be performed at the time the material is collected. Bottles or test tubes may be labeled with either wax pencil or some type of adherent label suitable for inscription. Blood slides may be labeled very readily by writing across the thick part in pencil; another method is to scratch the identification with the puncture point in very small letters across the beginning end of the blood film. In such manner a permanent label is obtained. Individual pipets may be labeled with special tubing, or the laboratory request slips may be attached to the pipets.

NUMERICAL COUNTING OF ERYTHROCYTES AND LEUKOCYTES

1. Counts should be taken in the morning while the patient is still in bed. Violent movement, work, or exercise may provoke temporary pictures which are not normal. Food or prolonged fasting usually increases the count. If consecutive counts are made, they should be made at the same hour each day.

2. Assemble the following material:

   (a) Sterile puncture point
   (b) Ether and alcohol
   (c) Red and white counting pipets, which should be dry
   (d) Red counting solution and white counting solution, previously filtered
   (e) Small tubes or watch glasses for holding the counting solutions

Preparation of patient: Cleanse the ear lobe with ether to produce hyperemia. If alcohol is used, it may cause the cells to change shape. Ether cleanses and at the same time produces hyperemia. If the finger is to be used, disinfect the skin with ether or alcohol and dry. If venous blood is used, transfer to special anticoagulant and mix thoroughly to prevent clotting.

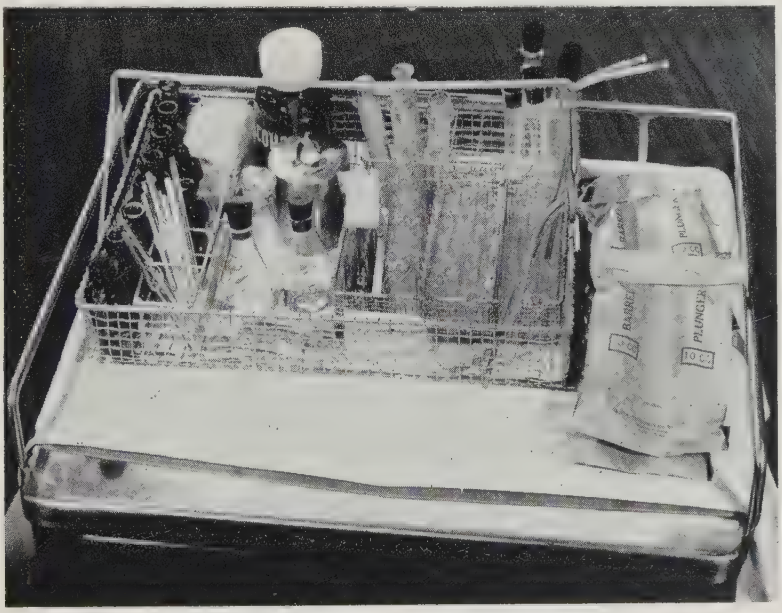

Fig. 347. Hospital blood procurement tray. Materials are carried on a suitable cart with wheels. Wire carrying basket (courtesy A. S. Aloe Co., St. Louis, Mo.). "Duets" sterile wrapping containers for syringes (courtesy Central States Paper & Bag Co., St. Louis, Mo.). Hemolets for finger puncture, sterile needles, cotton balls and antiseptic, and appropriate containers for blood specimens, pipets for individual blood samples (courtesy Veterans Hospital and Jewish Hospital, St. Louis, Mo.).

Technic of puncture: This technic is explained on p. 1099.

NUMERICAL COUNTS OF ERYTHROCYTES

Equipment:

1. Blood specimen.
   This may be capillary or venous oxalated blood.

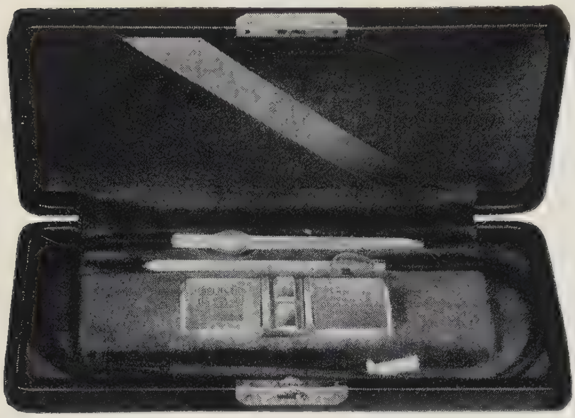

Fig. 348. Complete hemacytometer including improved double Neubauer counting chamber. (Courtesy American Optical Co., Scientific Instrument Division, Buffalo, N. Y.)

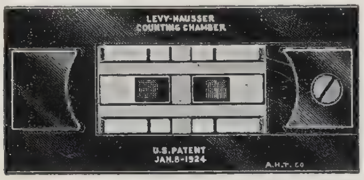

Fig. 349. Levy-Hausser counting chamber mounted in molded bakelite holder, forming a practically unbreakable unit. (Courtesy Arthur H. Thomas Co., Philadelphia, Pa.)

2. Red blood cell diluting pipet.
   There are 2 types of red counting pipet, the standard Thoma and the Trenner. The Thoma pipet illustrated in Fig. 354 has 10 divisions on the capillary end, with 0.5 and 1 points numbered prominently. If blood is drawn to the 0.5 mark and the pipet is filled with diluting fluid, the resultant dilution is 1:200 (1 vol. of the 101 total remains in the stem, does not enter the bulb, is blown out first before the counting chamber is filled, and therefore does not dilute the blood). This is the dilution used in routine counting. If the patient is quite anemic, a lesser dilution may be made by drawing the blood to the 1 mark and then filling the pipet with diluting fluid, a dilution of 1:100. Obviously, various dilutions may be obtained by using the different divisions on the capillary or stem for measuring the amount of blood to be used. The Trenner or automatic pipet is so constructed that when blood is drawn into the capillary end it will be carried by capillary attraction to a given point in the capillary. These pipets permit only 1 dilution, 1:200.
3. Hemacytometer.
   A counting chamber basically consists of a glass plate upon which have been etched lines, permitting accurate measurement of small volumes of blood. Fig. 348 illustrates one such hemacytometer. Fig. 351 shows the ruling of a single counting chamber. The most commonly used ruling is the improved Neubauer (Fig. 350, *5*). This ruling has been reproduced in Fig. 353. Note that a single chamber (of which there are usually 2 per hemacytometer) consists of 9 large squares, each measuring 1 sq.mm. Each of these squares covers a low-power field (16 mm. objective, 10× ocular). The central square of the chamber is subdivided into 25 smaller squares (each $\frac{1}{25}$ sq.mm.), 5 of which are marked **R** in Fig. 353. Each of these 25 squares is further

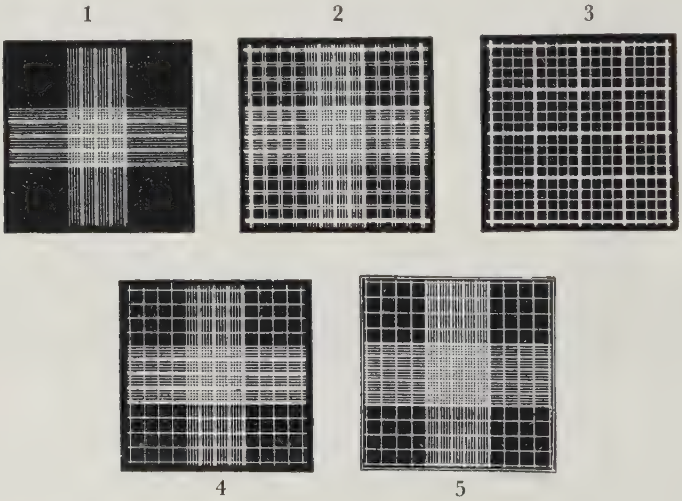

Fig. 350. Hemacytometer counting chamber rulings: **1**, Thoma; **2**, Tuerk; **3**, Fuchs-Rosenthal; **4**, Neubauer; **5**, improved Neubauer. (Courtesy E. H. Sargent Co., Chicago, Ill.)

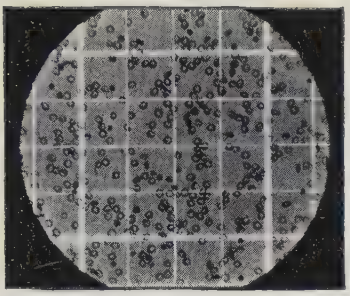

Fig. 351. Bright line counting chamber. (Courtesy American Optical Co., Scientific Instrument Division, Buffalo, N. Y.)

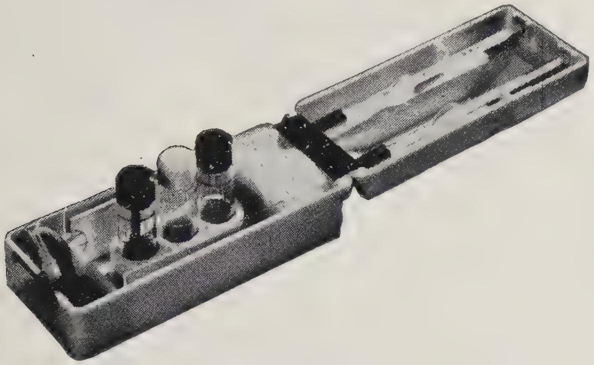

Fig. 352. Bard-Parker hematologic case.

divided into 16 squares ($\frac{1}{400}$ sq.mm. each). The 5 squares marked **R** are used for routine erythrocyte counting. A magnification of one of these **R** squares containing erythrocytes is illustrated in Fig. 351. One of these $\frac{1}{25}$ sq.mm. fields covers a high dry (4 mm. objective, 10× ocular) field.

Formula for determining factors in hemacytometry:

$V = D \times Dp \times S.A.$

V is the volume of blood actually measured.

D is the dilution of the blood sample (variable).

Dp is the depth of the counting chamber ($\frac{1}{10}$ mm.).

S.A. is the surface area counted (variable).

The formula for correcting counts to the number of cells/c.mm. whole blood is as follows:

$$C = N \times \frac{1}{V} \text{/c.mm.}$$

C is the cell count per c.mm.

N is the number of cells actually counted in the chamber.

V is the value from the formula above.

Example of how to use these formulas is given on p. 1105.

These formulas are applicable to any cell counting performed in a hemacytometer.

4. Hemacytometer cover glass.
   This is especially designed for this purpose. Do not substitute ordinary laboratory cover slips.
5. Microscope.
6. Carrying case for hematologic outfit.
   It is frequently necessary to collect blood for red and white counts outside the laboratory, hospital, or physician's office. Under these circumstances, the use of the Bard-Parker hematologic case* or some similar device is recommended (Fig. 352).

Reagents:

1. Diluting fluid. Hayem red cell counting solution.

Mercuric chloride, c.p. . .	0.5	gm.
Sodium sulfate, c.p., crystalline	5	gm.
or anhydrous	2.65	gm.
Sodium chloride, c.p. . .	1	gm.
Distilled water	200	ml.

Dissolve all the chemicals in the distilled water and filter several times through the same filter paper. Make a sufficient supply for only 2-3 wk., since deterioration might occur beyond this time, although Hayem solution is fairly stable. If a precipitate forms, discard the solution. This solution must be used at room temperature to prevent clumping of the erythrocytes due to the cold. **Clumping of the erythrocytes** may also occur in diseases associated with hyperglobulinemia, such as multiple myeloma, lymphomas, etc. It has been recommended that 0.01 gm. gelatin be added to each 100 ml. solution to prevent such agglutination.[82]

2. Gower diluting fluid.

Glacial acetic acid . . .	16.65	gm.
Sodium sulfate, c.p., crystalline	6.25	gm.
Distilled water	100	ml.

Add the ingredients slowly to the distilled water and mix thoroughly. It has been claimed[83] that Gower solution prevents rouleaux formation of the erythrocytes.

Taking red cell count:

1. If capillary blood is obtained from a skin puncture, wipe off the first drop with a piece of dry sterile cotton and use successive drops obtained without undue squeezing of the part, working rapidly to prevent clotting of the blood. If oxalated blood is used, it must be thoroughly mixed prior to use, preferably mechanically on a tube rotator.†
2. Make the dilutions of blood as perfectly as possible.
3. Holding the pipet **up** at an angle of 45° in the drop of blood and not touching the skin, draw the blood to exactly the 0.5 mark of the counting pipet (for routine counts). Always draw the blood into the pipet; never allow it to run in by capillary attraction.
4. Wipe the tip of the pipet clean of blood with a piece of dry cotton, gauze, or filter paper, without touching the opening of the capillary, and then immerse in the freshly filtered diluting fluid, which should be in a small tube or a watch glass. Do not insert the pipet in a bottle of counting solution.
5. Draw the diluting fluid steadily into the pipet by gentle mouth suction to exactly the 101 mark past the bulb of the pipet, rotating the pipet on its long axis to ensure thorough mixing of blood and diluent and gradually getting the pipet into a vertical position so that the measurement at the top of the pipet

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*Bard-Parker Co., Inc., Danbury, Conn.

†Baltimore Biological Laboratory, Baltimore, Md.

may be accurate. Do not permit the capillary tip of the pipet to come out of the diluting fluid while drawing up the solution, and do not permit any of the blood to run out of the capillary while filling the pipet. If the diluting fluid has been permitted to go beyond the 101 mark, or if air bubbles are present in the pipet, discard the mixture and begin again with a clean, dry pipet.

6. Immediately mix the contents of the pipet thoroughly by placing the thumb over one end and the first finger over the other end and shaking for a minute or so.

The diluted blood may be transported to the laboratory for further examination. Take care that none of the fluid escapes from the pipet. Lay the pipet on its side and avoid touching the tip to any object. Various devices including wide rubber bands and pipet tubing have been used for sealing the pipet. Pipet closures are available commercially. If rubber bands are used, seal off the small capillary end of the pipet first to prevent expulsion of fluid. In removing the device, reverse the procedure. When picking up the pipets for manual shaking, follow the same order of holding the pipets.

Diluted blood samples may be examined within 2 hr. However, these must be thoroughly mixed just prior to their admission into the hemacytometer.

Filling the counting chamber:

1. Shake the pipet for 2 or 3 min., holding it in one hand and striking it gently against the opposite hand. Mechanical shakers are available commercially and are highly desirable, particularly in a laboratory doing a large volume of work.
2. Thoroughly cleanse the hemacytometer and cover slip and render dry and fat-free. After use, wash in running cold water and wipe dry with fine gauze, lens paper, or linen. Avoid touching surfaces which are to come in contact with the blood specimen.
3. Place the cover slip in its proper position over the ruled areas of the hemacytometer and examine the assembled instrument for cleanliness under the low power of the microscope.
4. Discard the first 3 or 4 drops of diluted blood from the pipet and then fill the counting chamber by capillary attraction by placing the tip of the pipet at one of the open ends of the chamber. The angle of the pipet and the time required for filling the chamber vary with the speed of the pipet and can be determined only by experience. Carefully fill the counting chamber exactly. Overfilling can be readily observed by the presence of fluid in the moats surrounding each ruled area. If this occurs, clean the instrument and refill the counting chamber. An insufficient amount of fluid may be noted by the failure of fluid to cover a ruled area. Rectify by adding more fluid to the chamber. If air bubbles are present in the chamber, they usually indicate lack of cleanliness of the hemacytometer or cover slip or moisture. Clean again and refill the chamber.

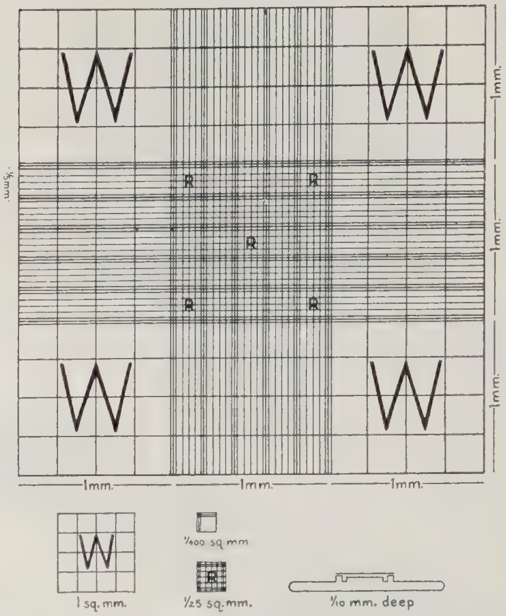

Fig. 353. Counting chamber rulings and dimensions.

5. Fill both ruled areas successively.
6. Allow the mixture to settle in the chamber. Locate the ruled area for erythrocyte counting under low magnification and then change to the high dry objective for actual counting of the cells.

Making the count: In a routine erythrocyte count, enumerate the cells in the five **R** squares (Fig. 353). Since Hayem solution does not dissolve leukocytes, do not count such cells in the red blood cell count. Leukocytes are larger than erythrocytes, appear grayish, and are usually granular. Only one such cell is seen in approximately 750-1000 erythrocytes; therefore leukocytes do not present a problem in routine red cell counting. When the white count is very high, particularly in leukemia, it is very important to make this differentiation.

A standardized pattern of counting should be adopted. It is recommended that counting begin in the left upper corner of each **R** square and proceed as follows. Count the first row of small subdivisions to the right, drop down to the second row and count to the left, drop down to the third row and count across to the right, and then finish the fourth row counting to the left. In the enumeration include all cells touching or half in and half out of the upper line and the line to the left, excluding those half in and half out of the lower and right-hand lines. Take care not to count any cell twice and do not omit any cells. **Remember that the boundary line is the center line of the triple ruling.** Count the first **R** square at the upper left of the ruling, the second at the upper right, the third at the lower right, the fourth at the lower left, and the fifth in the center (Fig. 353).

Record the count in each **R** square and obtain the sum of the cells in the 5 squares. (Mechanical counters are available commercially.)

Count 2 such chambers and take the average.

Calculation:

Example for routine counting: Multiply the sum of the 5 squares by 10,000.

454 cells counted in 5 **R** squares in 1 chamber.
456 cells counted in 5 **R** squares in the second chamber.

2) 910

455 cells counted in an average of 5 **R** squares.

Dilution of the blood 1:200. Area counted $\frac{5}{25}$ or $\frac{1}{5}$ sq.mm.

$V = \frac{1}{200} \times \frac{1}{10} \times \frac{1}{5} = \frac{1}{10{,}000}$ c.mm.

$$C = 455 \times \frac{1}{\frac{1}{10{,}000}}$$

$= 455 \times 10{,}000$

$= 4{,}550{,}000$ erythrocytes/c.mm. blood.

For anemic blood: Blood was drawn to the mark 1 in the pipet, making the dilution of blood 1:100.

455 cells were counted in the 5 **R** squares.

$V = \frac{1}{100} \times \frac{1}{10} \times \frac{1}{5}$ c.mm. $= \frac{1}{5{,}000}$ c.mm.

$$C = 455 \times \frac{1}{\frac{1}{5{,}000}} = 455 \times 5{,}000 = 2{,}275{,}000$$

erythrocytes/c.mm.

For extremely anemic blood: Blood was drawn to the mark 1 in the pipet, making the dilution of blood 1:100.

455 cells were counted in 10 **R** squares in each drop.

$V = \frac{1}{100} \times \frac{1}{10} \times \frac{10}{25} = \frac{1}{2{,}500}$ c.mm.

$$C = 455 \times \frac{1}{\frac{1}{2{,}500}} = 455 \times 2{,}500 = 1{,}137{,}500$$

erythrocytes/c.mm. blood.

For polycythemic blood: Blood was drawn to the 0.4 mark in the pipet, making a dilution of 0.4:100 or 1:250.

600 cells counted in 5 **R** squares.

$V = \frac{1}{250} \times \frac{1}{10} \times \frac{1}{5} = \frac{1}{12{,}500}$ c.mm.

$$C = 600 \times \frac{1}{\frac{1}{12{,}500}} = 600 \times 12{,}500 = 7{,}500{,}000$$

erythrocytes/c.mm. blood.

During the counting process observe the distribution of the erythrocytes. If clumps of cells are seen, the distribution is unsatisfactory, and an entirely new dilution of blood is required. If motion continues in the preparation in a streamlike fashion, counting is inaccurate. Such motion may result from overfilling or underfilling the chamber or from drafts caused by fans or open windows. These features must be corrected or eliminated.

Observations in the counting chambers should not be limited to merely tallying the numbers of red blood cells. Examine the relative size and shape of the erythrocytes. Normally there is uniformity of size and shape of the cells. In pathologic blood, lack of uniformity is often apparent.

As soon as the count has been made, clean the hemacytometer and cover slip in cold running water and dry with a clean lint-free cloth or lens paper. Do not allow preparations in the counting chamber to dry and do not perform counts on specimens which are drying. Clean the pipets immediately, or if that is impossible, empty them and fill at once with distilled water until such time as they can be cleaned.

Normal:

Males: 4,600,000-6,200,000/c.mm.; average 5,400,000

Females: 4,200,000-5,400,000/c.mm.; average 4,800,000

The normal values in children are somewhat different.

NUMERICAL COUNTS OF LEUKOCYTES

Equipment:

1. Blood specimen.
   This may be capillary or venous oxalated blood.
2. White blood cell diluting pipet.
   There are 2 types of pipets, the classic Thoma and the Trenner. The Thoma pipet, (Fig. 354) has 10 divisions on the capillary end with 0.5 and 1 prominently scored. Beyond the bulb is another mark, 11, which indicates the level to which diluting fluid is drawn. If blood is drawn to the 0.5 mark, the resultant dilution is 1:20 (1 vol. of the total 11 vol. remains in the stem and is blown out before the counting chamber is filled, leaving a total of 10 vol. diluted blood). For routine leukocyte counting a dilution of 1:20 is used. If marked leukopenia is present, a lesser dilution may be obtained by drawing blood to the 1 mark, a dilution of 1:10. Various dilutions to suit particular conditions may be obtained by drawing blood to any of the other divisions on the capillary pipet. The Trenner or automatic pipet permits of only 1 dilution, 1:20.
3. Hemacytometer. This is described on p. 1102.
   The four corner squares marked **W** in Fig. 353 are counted routinely. These are 1

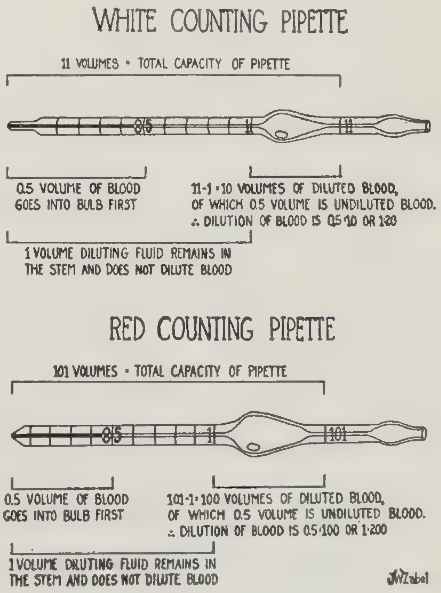
WHITE COUNTING PIPETTE
11 VOLUMES • TOTAL CAPACITY OF PIPETTE
0.5
1
11
0.5 VOLUME OF BLOOD GOES INTO BULB FIRST
11-1=10 VOLUMES OF DILUTED BLOOD, OF WHICH 0.5 VOLUME IS UNDILUTED BLOOD. ∴ DILUTION OF BLOOD IS 0.5:10 OR 1:20
1 VOLUME DILUTING FLUID REMAINS IN THE STEM AND DOES NOT DILUTE BLOOD
RED COUNTING PIPETTE
101 VOLUMES • TOTAL CAPACITY OF PIPETTE
0.5
1
101
0.5 VOLUME OF BLOOD GOES INTO BULB FIRST
101-1=100 VOLUMES OF DILUTED BLOOD, OF WHICH 0.5 VOLUME IS UNDILUTED BLOOD. ∴ DILUTION OF BLOOD IS 0.5:100 OR 1:200
1 VOLUME DILUTING FLUID REMAINS IN THE STEM AND DOES NOT DILUTE BLOOD

Fig. 354. Explanation of dilution of blood in red and white counting pipets.

sq.mm. each. One square covers a low-power dry objective field with a 10× ocular (16 mm. objective). The formula for determining factors in hemacytometry is given on p. 1103.

4. Hemacytometer cover glass (use no substitute).
5. Microscope.

Reagents:

1. Diluting fluid—1 or 2% acetic acid.
   Use 1 ml. (or 2 ml.) glacial acetic acid for each 100 ml. counting solution. Filter 3 times through the same filter paper. The solution must be made fresh at least once a week. The addition of a few drops of methylene blue or brilliant cresyl blue to 100 ml. of the solution will stain the leukocytes and aid in visualizing them under the microscope. Filter again immediately before use.
2. N/10 Hydrochloric acid.
   This diluent is used routinely when the Haden-Hausser hemoglobinometer is used. No dye should be added to this solution. Method of making normal solutions is given on p. 15.

Procedure:

1. Prepare the patient as directed on p. 1101 and make the puncture. Wipe off the first drop of blood.
2. Draw the blood to mark 0.5 (for routine counting) in the capillary end of the pipet and the counting solution to mark 11. Use the same technic as described for red blood cell counting. Be sure to wipe the blood off the outside of the pipet before immersing the pipet in the watch glass containing the counting solution.
3. Shake as for a red count, allow several drops to leave the pipet, and immediately fill both sides of the counting chamber.
4. Focus under low-power dry magnification, preferably with the condenser removed or lowered. Adjust the light so that the leukocytes appear as slightly iridescent round bodies with a very definite outline. If there is doubt as to whether an object is a leukocyte or an artifact, look at it under high magnification. Leukocytes have definite cell outlines and well-defined nuclear structure. Platelets appear as very small refractive bodies about one-fifth to one-seventh the size of the leukocytes, with no nuclei and slight brownian movement. Erythrocytes should not be present because the diluting fluid should hemolyze them. Occasionally ghost outlines of red cells may be seen.
5. For routine counting, enumerate the cells in the 4 corner **W** squares, each divided into 16 smaller squares for convenience in counting. Count first the upper left **W** square of 16 small squares, then the upper right, the lower right, and last the lower left. The enumeration should follow the same pattern described under erythrocyte counting. Include in the count those leukocytes which are half in and half out of the upper and left-hand lines, but not those half in and half out of the lower and right-hand lines. Remember that the boundary line is the center line of the triple ruling.
6. Count 2 such drops, one in each side of the counting chamber.
7. Record the counts and obtain the sum. Or use a mechanical counter.

Calculations:

For routine leukocyte counts: Multiply the count of 4 **W** squares by 50.

194 cells counted in the first count.
198 cells counted in the second count.
2) 392 cells
196 leukocytes counted in 4 **W** squares.

Dilution of the blood 1:20; area counted 4 sq.mm.

$V = \frac{1}{20} \times \frac{1}{10} \times 4 = \frac{1}{50}$ c.mm.

$C = 196 \times \frac{1}{\frac{1}{50}} = 196 \times 50 = 9{,}800$ leukocytes/c.mm.

Comment: **In routine leukocyte counting only,** in which 4 **W** squares are counted, the mathematics may be simplified by dividing the number of cells counted in a total of 4 **W** squares by 2 and adding two 0's. An average of the 2 counts must be made.

Example for leukopenic blood:

98 leukocytes counted in 8 **W** squares

Dilution of blood 1:10 (blood drawn to mark 1 instead of 0.5)

Area counted 8 sq.mm.

$V = \frac{1}{10} \times \frac{1}{10} \times 8 = \frac{8}{100}$ c.mm.

$C = 98 \times \frac{1}{\frac{8}{100}} = \frac{9{,}800}{8} = 1225$ leukocytes/c.mm.

Example for blood with leukocytosis:

600 cells counted in 4 **W** squares (4 sq.mm.).

Dilution of blood 1:33 (blood drawn to 0.3 mark in pipet).

$V = \frac{1}{33} \times \frac{1}{10} \times 4$ c.mm. $= \frac{4}{330}$ c.mm.

$C = 600 \times \frac{1}{\frac{4}{330}} = \frac{600 \times 330}{4} = 49{,}500$ leukocytes/c.mm.

Normal:

Adults: 4500-11,500/c.mm.; average 7400

Comments: In instances in which leukocyte counts exceed 50,000/c.mm., dilutions may be made in the red cell diluting pipet, using white counting solution. If blood is drawn to the 1 mark in the red pipet, the dilution is 1:100.

If a specimen is taken routinely in the white counting pipet and it is found that the count is too high to enumerate accurately, and if the patient is not available for another dilution to be made, count the cells under high magnification in the center square (the one divided into 400 small squares), counting all the cells in the entire 25 squares or 1 sq.mm. Do not take an average. The area counted is 1 sq.mm., the dilution of blood is 1:20, and the depth of the counting chamber is $\frac{1}{10}$ mm.

Multiply the sum of the cells by 200.

During the counting process, observe for equality in distribution of the cells. If clumps are present, the distribution is unsatisfactory, and an entirely new dilution of blood is required.

As soon as tallying has been completed, clean the hemacytometer and cover slip in cold running water and dry with a clean lint-free cloth or lens paper. Do not allow specimens in the counting chamber to dry and do not perform counts on preparations which are drying. Clean the counting pipets immediately, or if that is not possible, empty them and fill with distilled water until such time as they can be cleaned.

Historical: The procedure of counting the total number of white cells in the blood seems to have been initiated by Malassez in 1872.[84] The method of studying the white and red cells was worked out further by Gowers in 1877; by Thoma in 1882, who used the red pipet and modified it for the leukocytes, thus giving us the pipet so universally used ever since; by Alferow in 1884, who distinguished white cells by raising the tube to lengthen focal distance; and by Toisson in 1885, who used a fluid permitting the counting of white cells in the same pipet with red cells. This ideal was pursued by Schüffner further in 1911 and Türk in 1912. Zappert in 1892 worked out the first satisfactory ruling for the counting chamber. Rieder in 1892, Elzholz in 1894, Friedlander in 1897, Durham in 1897, Einhorn and Laporte in 1902 (white count being made directly from film, by noting the ratio of white cells to red cells and counting only the red cells in chamber), Türk in 1902, Breuer in 1902, Joffroy and Mercier in 1902, Engel in 1902 (differentiation of white cells by refraction of light, most like Alferow), Burker in 1905 and in 1907, Ellermann and Erlandsen in 1909, Dunger in 1910, Galambos in 1910, Hill in 1912, and Roderdansz in 1913 further worked out the details of counting white cells.

Making numerical counts of leukocytes in blood showing large numbers of nucleated red cells

There are some conditions accompanied by an influx of nucleated red cells into the blood stream. When leukocyte counts are made of such bloods, it is often difficult and sometimes impossible to differentiate under low magnification between leukocytes and nucleated red blood cells that are not dissolved by acetic acid. These cells might therefore be included in the total leukocyte count. If the peripheral spread shows many nucleated red cells, the total leukocytes counted should be corrected for their number.

Correcting leukocyte count for nucleated red blood cells: Determine the number of nucleated red cells/100 white blood cells in the peripheral blood film and then use the formula in the example below to determine X, which must be subtracted from the uncorrected total white blood cell count in order to give the corrected white blood cell count.

The corrected leukocyte count = the total uncorrected leukocyte count − X (the absolute number of nucleated RBC/c.mm.). The value for X is calculated as follows:

$$X = \frac{\text{No. nucleated RBC/100 uncorr. WBC}}{100 + \text{No. nucleated RBC/100 uncorr. WBC}}$$

× Uncorr. WBC/c.mm.

Example:

Uncorrected blood count = 19,500

25 nucleated RBC/100 actual cells as counted on blood film

$$X = \frac{25}{100 + 25} \times 19{,}500 = 3900$$

Corrected WBC count = 19,500 − 3900 = 15,600.

CLEANING THE PIPET

Eject the contents of the pipet immediately after using. Never allow the blood to remain in the pipet, as this might cause permanent blockage. Use a suction pump to clean the pipet, drawing the following various solutions through it.

Wash thoroughly with distilled water until all traces of blood have been removed. Next, draw alcohol through the pipet to remove the water. Next, draw ether through the pipet to remove the alcohol and to dry. Continue the use of suction until the pipet is thoroughly dry, as evidenced by the jumping of the glass bead in the bulb. If the glass bead adheres to the side of the bulb, the pipet still contains moisture which must be removed with alcohol and ether.

Ammonia water, potassium hydroxide, or N/10 hydrochloric acid may be used in cleaning the pipet, provided that all traces of the chemical employed are removed before the pipet is used again.

Some laboratories prefer to clean the pipets with distilled water, followed by acetone drawn through the pipets, and then an air current to dry them.

Caution! If the pipets are set aside after the dilutions are made, the contents must be thoroughly mixed before filling the counting chamber, as the cells will tend to settle at the bottom of the bulb, and subsequent counts without mixing will be inaccurate.

In making counts, count all cells within the ruled area and in addition all cells which lie along the ruled lines at the top and left side of the field. Omit all cells lying on the bottom and right sides unless they are definitely within the ruled area. Remember that the center line of the triple ruling is the border of the field in some counting chambers. One must study the rulings to distinguish between guide lines and boundaries of fields.

PRECAUTIONS TO BE OBSERVED IN MAKING RED AND WHITE COUNTS AND BLOOD FILMS

1. Always remove the first drop or two of blood that oozes out after the skin puncture. Always remove the blood that is left between taking of the various specimens, such as red and white counts, films, etc.
2. Never under any circumstances squeeze to obtain blood.
3. Hold the pipets either vertically or up at a 45° angle; never allow them to hang down from the puncture.
4. If blood clots occur in the red and white counts, in spreads, thick drop, or hemoglobin test, repeat the preparations to avoid inaccuracies.
5. Be sure to draw the blood for red and white counts and hemoglobin exactly to the mark on the pipet—never above nor below.
6. Be sure that no air bubbles enter the pipet during the diluting process.
7. Always be sure there is no blood on the outside of the pipet before drawing up the counting solution.
8. Shake the pipets for several minutes before filling the counting chamber, but do not shake them too hard.
9. Eject several drops of diluted blood from the pipet before filling the counting chamber. If more than 1 drop of the dilution is counted, the pipet must be shaken before filling the counting chamber the second time, and several drops of the dilution must be ejected.
10. Never overfill nor underfill the counting chamber. If air bubbles are present, clean the counting chamber and refill. If the counting chamber is imperfectly overfilled, clean the counting chamber and refill with a fresh drop.
11. It is well to take the specimens for the complete blood picture in the following order: films, white count, red count, thick drop, and hemoglobin.
12. Under no circumstances allow the pipets for red and white counts and hemoglobin or the slides for films and thick drops to touch the skin.
13. When diluting the red and white counts, be sure that no blood runs back out of the pipets while drawing up the diluting fluid.
14. Keep blood films and thick drops out of dust and away from flies and other insects.
15. Always make the counts as soon after taking the specimen as possible.
16. Use fresh counting solution for each count. Filter enough solution into a watch glass or small tube to be used for diluting 1 count. Do not dip the pipet into the stock bottle of counting solution.
17. Never use oxalated or heparinized blood for making blood films.

The sources of error of red and white cell counting are numerous. Some can be eliminated by adherence to the points just mentioned; others are inherent to the method and can be minimized only by counting large numbers of cells, e.g., 2000.

There are errors which are due to the methods of collecting and handling of the blood, whereas others are due to the equipment and its handling, and finally some are statistical errors, as when a small quantity is used to represent a large mass. The average error of the red cell count, using excellent technic, is ± 11%. This error may increase to ± 22%.

In the white cell count, in which the usual dilution is not as great as in the red cell count, the error is about ± 15%.

Because of the relatively large possible error of the method, each laboratory should establish a standard deviation of these methods in order to better evaluate the accuracy of blood counts and to better interpret fluctuating repeated counts on a given patient.

The method of calculating the standard deviation as a means of evaluating the variations of results of a particular method applies to all laboratory work and is not restricted to hematology.

ELECTRONIC COUNTERS[35-39]

An electronic counter* counts the individual red blood cells of a measured volume of diluted blood as they pass through a minute orifice guarded by an electric current flowing between platinum electrodes. The red blood cells, which are poor electrical conductors, in passing through the orifice displace an electrically conductive 0.9% saline solution in which they are suspended and thus modulate the electric current.

The modulations are amplified and counted.

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*Coulter Electronics, Chicago, Ill.

They depend on the number and size of the cells passing through the orifice. The counter works with an accuracy and reproducibility of about 2%. The electronic counter enumerates red and white blood cells alike, and an error will be introduced if leukocytosis of over 30,000 cells/c.mm. exists. In this case the result must be corrected. The electronic counter can also be used in enumerating leukocytes and platelets and accomplishes that with a standard error of 2.8%.

Recently the use of a stable suspension of latex particles of known size and concentration has been described as a device for monitoring electronic counting instruments.

HEMOGLOBIN DETERMINATIONS

Hemoglobin is an iron-bearing protein which is the red coloring matter of the blood. Discussion of hemoglobin is found on pp. 1080 and 1081.

Normal:

Adult males: 14-18 gm./100 ml. blood; average 15.8.

Adult females: 11.5-16 gm./100 ml. blood; average 13.9.

METHODS IN HEMOGLOBINOMETRY

There are 4 basic methods of measuring hemoglobin content of the blood.

A. Colorimetric
   1. Hemoglobin examined directly as oxyhemoglobin by the methods of Dare, Tallqvist, and Spencer instrument and the photoelectric method
   2. Hemoglobin converted to acid hematin by the methods of Sahli, Newcomer, Haden-Hausser, and LaMotte and the photoelectric method
   3. Hemoglobin converted to alkaline hematin in photoelectric methods
   4. Hemoglobin converted to CO-hemoglobin by the method of Haldane
   5. Hemoglobin converted to cyanmethemoglobin
   6. Hemoglobin converted to pyridine hemochromogen
   7. Hemoglobin directly matched with artificial standards

B. Physical
   1. Physical measurement of specific gravity of whole blood and serum or plasma by the copper sulfate method
   2. Hematocrit

C. Gasometric measurement of oxygen capacity of blood (method using Van Slyke apparatus)

D. Measurement of iron content of blood by chemical means

The methods are further subdivided into those that lend themselves to routine clinical use and those that are accurate enough to be utilized for checking the accuracy of the routine clinical method (methods of standardization).

ROUTINE CLINICAL METHODS

Colorimetric methods

Dare method

Instrument is standardized so that 16 gm./100 ml. blood is the equivalent of 100% hemoglobin.

Principle: The Dare hemoglobinometer is based on the principle that the color of a thin film of undiluted blood illuminated by a standard light source may be compared with a graduated nonfading color scale built into the apparatus. Models are available in both battery or electrically operated light sources. The matching fields are supplied in 2 types, regular or juxtaposition fields.

Procedure:

1. Prepare the instrument by placing the 2 pieces of glass (1 frosted and 1 plain) together and inserting them into the small metal holder.
2. Make a puncture in the usual manner, discarding the first drop of blood. Allow the blood to run into the space between the 2 pieces of glass; then place the specimen in the instrument so that the white glass will be toward the source of light.
3. Look through the telescope, holding the hemoglobinometer in a position which allows the light to penetrate the blood specimen.
4. Revolve the standard by turning the knurled knob at the top of the instrument, until the

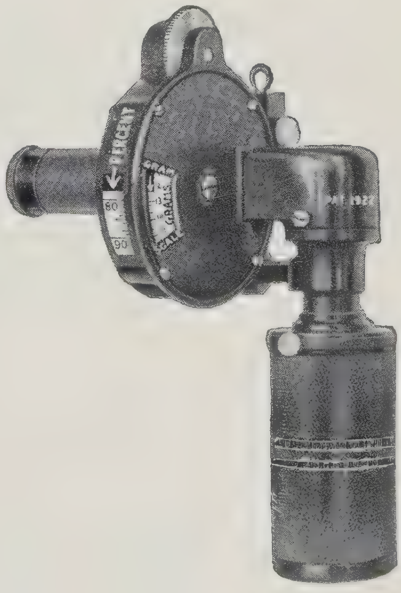

Fig. 355. Dare hemoglobinometer with gram scale. (Courtesy Rieker Instrument Co., Philadelphia, Pa.)

color of the unknown exactly matches that of the standard.
5. Read from the graduation marks at the side of the instrument the percentage or gm. hemoglobin.
6. To convert to gm. hemoglobin/100 ml. blood, multiply the percentage of hemoglobin by 16 or convert to gm. by reading directly off the scale.

Example:
Percentage of hemoglobin is 95.
0.95 × 16 = 15.20.
After use, the instrument should be cleaned and dried immediately.

This method of direct matching is subject to considerable error and is therefore not recommended.

Spencer hemoglobinometer

The Spencer* hemoglobinometer is a pocket-sized instrument operated by battery or electric current and is convenient for determining hemoglobin concentration. It utilizes light in the visible green spectrum. In addition to the hemoglobinometer, hemolysis applicators are needed. This method is subject to the errors of all direct matching methods.

Procedure:
1. Place a large drop of blood from a skin puncture directly onto the open glass chamber of the apparatus. Hemolyze it completely with a hemolysis applicator until blood change is apparent. This takes approximately 45 sec. Close the chamber by sliding it under the cover glass and insert into the instrument. The hemolyzed blood must entirely fill the chamber; there must be no air spaces.
2. Press the illuminating switch and observe through the eyepiece. There will be seen 2 halves of a circular field, one which is stationary and represents the standard and the other which is movable and represents the unknown. By moving the lever on the side of the instrument, the fields can be brought to match each other. It is well to take several readings until 2 consecutive readings are alike.
3. Read the hemoglobin concentration directly from the graduated scales on the side of the apparatus. There are 4 scales on the instrument: gm./100 ml., scale based on a standard of 15.6 gm./100 ml. blood, % scale on a basis of 14.5 gm., and % on a standard of 13.8 gm. The inclusion of 4 scale readings on this apparatus is magnificent testimony to the widespread confusion as to what level of hemoglobin concentration is normal.

Sahli method

The Sahli hemoglobinometer consists of a set of glass rods colored amber and is housed in a casing with a central aperture. Into this center fits a graduated tube with a scale reading in either grams of hemoglobin/100 ml. blood or percentage of hemoglobin. The Sahli pipet has a capacity of 20 c.mm. (0.020 ml.). N/10 hydrochloric acid is used to convert to acid hematin.

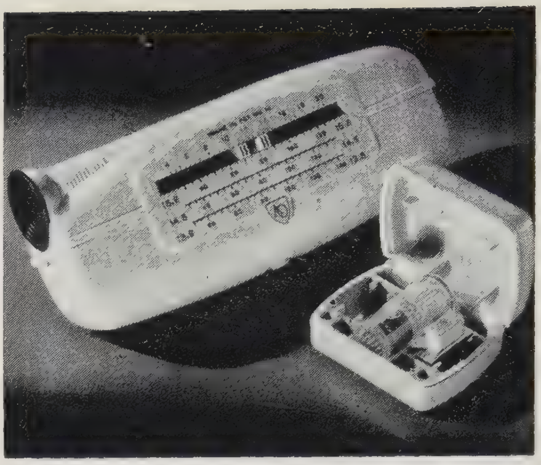

Fig. 356. Spencer hemoglobinometer. (Courtesy American Optical Co., Scientific Instrument Division, Buffalo, N. Y.)

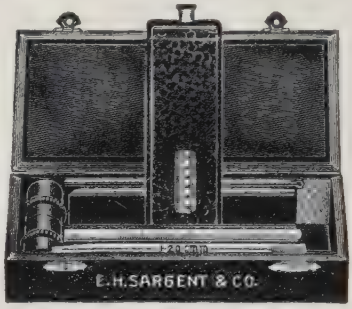

Fig. 357. Sahli hemoglobinometer. (Courtesy E. H. Sargent Co., Chicago, Ill.)

Procedure:
1. Prepare the instrument by placing N/10 hydrochloric acid in the graduated tube until the meniscus stands at mark 10 on the percentage scale.
2. Draw blood into the Sahli pipet to mark 20. Wipe the blood off the outside of the pipet.
3. Eject the blood into the hydrochloric acid solution and mix by drawing in and out of the pipet several times. Blow through the pipet while removing it from the tube.
4. Shake the tube and allow to stand a minimum of 1 min. and a maximum of 10 min.
5. Add distilled water a drop at a time, mixing after the addition of each drop, until the color in the tube corresponds exactly to the color of the standard. Do not go beyond the point at which exact color matching has occurred. If this happens, repeat the entire procedure.
6. Read in north daylight and turn the gradua-

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*Scientific Instrument Division, American Optical Co., Buffalo, N. Y.

tions to the side in order to read through clear glass.

7. Read from the scale the percentage of hemoglobin. To convert to gm./100 ml. blood, multiply the percentage figure by the gram standard on the tube.

Normal: Sahli instruments have been on the market calibrated so that the normal values were 100% equivalent to 13.8, 14, 14.5, 17, 17.2, and 17.3 gm. hemoglobin/100 ml. blood.

Comment: This acid hematin method is simple. The apparatus is relatively inexpensive. The variety of standards is confusing, and the visual matching is subject to considerable individual variation. The pigment formation progresses at a variable speed, and the pigment forms a suspension rather than a solution. These criticisms also apply to the other acid hematin methods, such as the Newcomer and Haden-Hausser.

Newcomer method

The equipment consists of a standard Newcomer colored disk. Note the thickness inscribed on the disk. A Klett or Duboscq type colorimeter and a Sahli hemoglobin pipet are used.

Procedure:

1. Place the standard Newcomer disk in the Duboscq or Klett colorimeter preparatory to the reading of the hemoglobin test.
2. Place 5 ml. N/10 hydrochloric acid in a colorimeter cup.
3. Draw the blood to the 20 mark in a Sahli pipet.
4. Eject the blood into the hydrochloric acid and mix by drawing in and out of the pipet several times. Note the time at which the blood enters the acid solution.
5. Mix the contents of the cup well and allow to stand 10 min.
6. Have the cup below the standard disk filled with distilled water. Be sure the plunger enters the distilled water.
7. Match the colors in the colorimeter and obtain the reading. Calculate the gm. hemoglobin/100 ml. blood by dividing the reading in Table 227 by the reading on the colorimeter.

Table 227 takes into account the time which has elapsed before the reading was made and the thickness of the glass standard. The exact thickness of the glass disk is engraved on it.

Example:

10 min. elapsed before the reading was made.
Thickness of glass disk was 1 mm.
Reading on the colorimeter was 6.7.
99.4 ÷ 6.7 = 14.83.

Normal: The Newcomer standard is 16.92 gm. hemoglobin/100 ml. blood.

Haden-Hausser method[90]

The Haden-Hausser hemoglobinometer is manufactured in 2 models, the clinical and the laboratory. The basic principles and general design of the 2 instruments are essentially the same. The laboratory model is larger and features an electrically controlled light source. A miniature comparator scale graded from 7.5 to 19 gm. hemoglobin is built into a well in the apparatus. This is overlaid by a removable fitted glass cover slip which when in position directs the flow of the blood sample into the chamber.

A white blood cell counting pipet is used.

Procedure:

1. Dilute the blood 1:20 or 1:10 in a white blood cell counting pipet with N/10 hydrochloric acid.
2. Allow to stand at least 30 min. to complete conversion of the hemoglobin into acid hematin.
3. Run the diluted blood into the dilution channel of the comparator.
4. Move the comparator until a matching of the blood specimen and a colored segment of the comparator is found. Read values directly from the instrument.

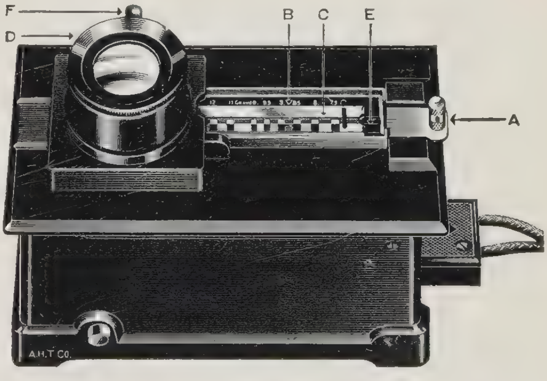

Fig. 358. Haden hemoglobinometer. (Courtesy Arthur H. Thomas Co., Philadelphia, Pa.)

Table 227

Minutes since dilution	Thickness of colored glass in ml.										
	0.95	*0.96*	*0.97*	*0.98*	*0.99*	*1.00*	*1.01*	*1.02*	*1.03*	*1.04*	*1.05*
10	93.4	95.4	96.4	97.4	98.4	99.4	100.4	101.4	102.3	103.4	104.4
15	93.1	94.1	95.0	96.0	97.0	98.0	99.0	100.0	101.0	102.0	103.0
20	92.5	93.5	94.5	95.4	96.4	97.4	98.4	99.4	100.4	101.3	102.3
30	91.8	92.8	93.8	94.8	95.7	96.7	97.7	98.6	96.6	100.5	101.5
40	91.6	92.5	93.5	94.5	95.4	96.4	97.4	98.3	99.3	100.3	101.2
Final	90.6	91.6	92.5	93.5	94.4	95.4	96.4	97.3	98.3	99.2	100.2

Normal: The Haden-Hausser standard is 16.7 gm. hemoglobin/100 ml. blood, which is equivalent to 100%.

Comment: An advantage of this method is that since the dilution of the blood is made in a white blood cell counting pipet both the leukocyte count and the hemoglobin estimation can be carried out with the same diluted blood specimen.

Photoelectric hemoglobin determinations based on measurement of oxyhemoglobin

The methods of determining hemoglobin by photoelectric colorimetry are given on p. 1109.

In the original method of Sheard and Sanford, which was the first photoelectric hemoglobin determination, 0.1% sodium carbonate was used as a diluent. The color of the resultant solution tends to fade within 3-4 hr. so that reading of the test must be made within this time limit. It has been found that 0.04% ammonium hydroxide will keep oxyhemoglobin solutions stable for as long as 4 days.[91] Hypotonic saline solutions have been generally abandoned as diluting fluids.

Normal: Normal values vary with the method and standardization of the photoelectric colorimeter. Values should preferably be expressed in gm. hemoglobin/100 ml. blood.

Photoelectric determination of hemoglobin based on measurement of acid hematin

The Fisher apparatus has an ammeter reading scale limited to percentage and gm.% hemoglobin. Any photoelectric colorimeter may be calibrated for this method. N/10 hydrochloric acid is used as the diluent. It takes about 30 min. for full development of the brown color; this time should be the minimum allotted for reading unknown samples.

Procedure:

1. Use either venous or finger blood.
2. Draw the blood into the hemopipet exactly to the graduation mark below the bulb and wipe the tip of the pipet clean.
3. Draw N/10 hydrochloric acid into the pipet to exactly the graduation mark above the bulb.
4. Allow the mixture to remain in the pipet for 30 min. or transfer it to the Fisher photometric cell and allow it to remain for 30 min.
5. Be sure that the pointer reads on the line marked **A.** Adjust by rotating the knob on the top of the instrument at the point marked **Adjust Zero.**
6. Turn the switch to **On** and allow the instrument to remain for 1-2 min. Insert the standardizing cell containing distilled water through the hole just in front of the reading scale. Turn the knob on the front panel and adjust the pointer to line **B.**
7. Remove the water-filled cell and replace with cell containing the blood specimen.
8. The pointer now indicates the hemoglobin concentration of the blood specimen both in gm. hemoglobin/100 ml. blood and in % normal.

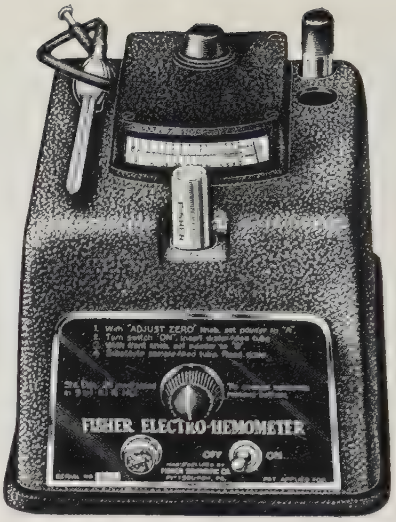

Fig. 359. Fisher Electro-Hemometer. (Courtesy Fisher Scientific Co., Pittsburgh, Pa.)

Normal: Normal values vary with the technic and standardization of the photoelectric colorimeter. The Fisher Electro-Hemometer (Fig. 359) uses a normal standard of 15.6 gm. hemoglobin/100 ml. blood.

Comments: This method has generally been discarded in favor of methods using oxyhemoglobin.

Methods in which hemoglobin is converted to alkaline hematin

These are photoelectric methods which basically measure dilutions of blood treated with alkali for conversion of hemoglobin to alkaline hematin. These dilutions are read photoelectrically, usually with a filter at 540 mμ. The instrument is calibrated against known solutions of hemoglobin. Graphs are prepared from the readings from known solutions, and unknown determinations are made from the graphs. Determination of hemoglobin based on alkaline hematin has not gained popularity and is rarely used. Fetal hemoglobin resists alkaline denaturation, and the alkaline hematin method is therefore not generally applicable.

Methods in which hemoglobin is converted to carbon monoxide hemoglobin (carbon monoxyhemoglobin)

The colorimeter may be the Duboscq type of visual colorimeter or a photoelectric instrument. This method employs artificial illuminating gas which is not generally available.

Reagents:

1. Ammonium hydroxide solution, 0.4%.
   Dilute 4 ml. ammonium hydroxide to 1000 ml. with distilled water in a liter volumetric flask.
2. Carbon monoxide.
   CO may be obtained by running a stream of illuminating gas through the specimen or by using commercially available tanks of carbon monoxide.

Normal: In the original Haldane methods the standard was 13.8 gm. hemoglobin/100 ml. blood.

Methods in which hemoglobin is converted to cyanmethemoglobin

Reagent:

1. Drabkin solution.[92, 93]

Sodium bicarbonate	1 gm.
Potassium cyanide	52 mg.
Potassium ferricyanide . .	198 mg.
Distilled water	1000 ml.

Ferricyanide oxidizes hemoglobin from ferrous iron to ferric iron to form methemoglobin in an alkaline medium. Methemoglobin interacts with potassium cyanide to form cyanmethemoglobin, a stable hemoglobin pigment. This reagent is available in pellet form* and is prepared by adding 1 pellet to 100 ml. distilled water.

Procedure:

1. Use a calibrated Sahli pipet to deliver 0.02 ml. capillary or venous oxalated blood to 5 ml. Drabkin solution in a dry cuvet. Rinse the blood out of the pipet repeatedly in the Drabkin solution. Mix by swirling the tube.
2. Allow the mixture to stand 10 min. If the mixture is turbid, add a pinch of saponin to hemolyze the red cells completely.
3. Use the diluent as a blank, set the wavelength at 540 mμ, and set the instrument at 100% transmission (% T) or zero optical density (OD).
4. Place the unknown into the instrument and record OD or T.

To find gm.% hemoglobin of the whole blood, refer to the standard curve. The standard curve can be prepared by using a commercially available cyanmethemoglobin standard.†

Comments: The cyanmethemoglobin method of hemoglobinometry has been recommended by a panel formed under the aegis of the National Research Council with a number of sponsoring groups.[94]

This method is precise and rapid. The results are somewhat on the high side of normal, as apparently other substances contained in blood besides hemoglobin react with potassium ferricyanide.

The use of a solution of cyanide in routine laboratory work poses the question of safety. Crosby et al.[95] take cognizance of this danger and recommend the following precautions:

"1. Pipets should be filled with a suction bulb.

"2. Blood should be mixed with the cyanide solution by swirling.

"3. If small amounts of cyanide compounds are accidentally spilled on the laboratory bench or on the floor during the weighing procedure, the dry powder should be wiped up with a damp cloth and discarded in a suitable container.

"4. Cyanide salts should be stored in a locked cupboard."*

The level of cyanide is far below the lethal dose. The lethal dose of Aculute is approximately 16 pellets to 4000 ml. diluent.

Calibration of cyanmethemoglobin method: A standard solution of cyanmethemoglobin is available commercially.† The concentration of cyanmethemoglobin is given in mg./100 ml. and is usually of the order of 60 mg. hemoglobin/100 ml. blood. The equivalence of this standard can be calculated as follows:

The dilution employed in the procedure just described is 1:251.

The equivalence of the standard in gm.% hemoglobin

$$= \frac{\text{Concentration Hb in standard} \times 251}{1000}$$

Example:

Concentration of hemoglobin in standard solution: 60.5 mg. Hb/100 ml.

$$\text{Equivalence of standard} = \frac{60.5 \times 251}{1000} = 15.2$$

gm.% Hb

Having calculated the value of the standard proceed as follows.

Prepare 5 cuvets: a blank tube containing 6 ml. diluent, tube 1 containing 6 ml. undiluted standard, tube 2 containing 4 ml. standard plus 2 ml. diluent, tube 3 containing 3 ml. standard plus 3 ml. diluent, and tube 4 containing 2 ml. standard plus 4 ml. diluent.

Determine the transmittance of tubes 1-4 using the blank tube as reference.

Make a semilogarithmic plot of % T obtained versus the hemoglobin equivalent of each dilution. The hemoglobin equivalent of each dilution is obtained by multiplying the hemoglobin equivalent of the undiluted standard by the appropriate dilution factor, i.e., tube 1 = 1.0, tube 2 = 0.666, tube 3 = 0.500, and tube 4 = 0.333.

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*Aculute; Ortho Pharmaceutical Corp., Raritan, N. J.

†Acuglobin hemoglobin standard; Ortho Pharmaceutical Corp., Raritan, N. J.

*From Crosby, W. H., et al.: U.S. Armed Forces M. J. 5:693, 1954.

†Ortho Pharmaceutical Corp., Raritan, N. J.

Methods in which hemoglobin determinations are based on specific gravity of whole blood and serum or plasma

This method, frequently referred to as the copper sulfate method, is a useful rough guide in estimating hemoglobin concentrations and total proteins simultaneously. As a screening process it is of inestimable value, particularly when large numbers of determinations must be made. The method was developed during World War II and was popularized in the Armed Forces of the United States. It is not recommended as a routine clinical procedure.

Copper sulfate method to determine specific gravity of blood and hemoglobin content[95a]

This method makes it possible with 3 or 4 drops of blood, a medicine dropper, and small bottles of copper sulfate solution to determine the specific gravity of the blood and from it the hemoglobin content within 10%. The plasma protein concentration may also be determined. To measure the blood and plasma specific gravities and calculate the plasma proteins, hemoglobin, and hematocrit on the line charts requires about 2 min.

Principle: The technic consists of letting drops of plasma or whole blood fall into a graded series of solutions of copper sulfate of known specific gravity and noting whether the drops rise or fall in the solutions. Each drop on entering the solution becomes encased in a sack of copper proteinate and remains as a discrete drop without change of gravity for 15 or 20 sec., during which its rise or fall reveals its gravity relative to that of the solution. The size of the drop does not have to be constant; hence no special pipet is needed. No temperature correction is required. The copper sulfate solution automatically cleans itself after each test because within 1 or 2 min. after the test is completed the material of the drop settles to the bottom as a precipitate. The standard copper sulfate solutions are prepared by dilution of a stock solution of gravity 1.1000, which can be prepared either from weighed amounts of $CuSO_4 \cdot 5H_2O$, or by dilution from a saturated solution of the sulfate. The latter procedure makes it possible to prepare the standard solutions without a balance.

For accurate work a series of copper sulfate solutions graded at intervals of 0.001 in specific gravity is used; 21 solutions cover the plasma range 1.015-1.035 and 41 cover the whole blood range, 1.035-1.075. For rougher work with gravities accurate to ±0.001, 16 solutions at intervals of 0.004 suffice to cover the entire range of blood and plasma

Apparatus:

1. Apparatus for drawing and preparing blood.
2. Centrifuge (can be omitted if one can wait for blood to clot or sediment until a few drops of serum or plasma can be obtained).
3. Medicine droppers.
4. Oxalated test tubes, heavy-walled Pyrex, of about 10 ml. capacity (125 × 16 mm.). These are prepared in advance for 5 ml. portions of blood by pipetting into each tube 0.25 ml. of a 2% solution of 3:2 ammonium-potassium oxalate (see Reagents). The solution is spread in a film about the lower half of the tube and dried in an incubator (not over 50° C.) or in a vacuum desiccator. The tube is marked on the outside to hold 5 ml.
5. Bottles or tubes for standard copper sulfate solutions: One or more of the following sets will be needed.
    - (a) Laboratory set covering the ranges of blood, plasma, and transudates. Seventy oval or round bottles of 4 oz. capacity. This number will hold solutions covering the range 1.008-1.075 at 0.001 intervals and provide 3 spares. With these intervals the gravities can be estimated to ±0.0002. The 100 ml. portions of solution held by these bottles suffice for analysis of about 100 blood specimens and their plasmas The stoppers should be rubber, cork, or screw cap rather than glass, to prevent "freezing" and evaporation.
    - (b) Portable set covering the same range. One oz. bottles with screw caps. The 25 ml. portions of solution held by these bottles suffice for analysis of about 25 blood specimens and their plasmas.
    - (c) Field set for approximate analyses covering the range of blood and plasma. This includes 16 bottles to hold solutions covering the range 1.016-1.074 at intervals of 0.004. It serves for gravities within ±0.001. The bottles may be 1, 2, or 4 oz., according to the number of specimens of blood to be analyzed and the space and weight desirable for the set.
    - (d) Pocket field set for whole blood. This consists of six 1 oz. bottles to hold solutions of gravities covering the range 1.040-1.070 at intervals of 0.006. It serves to estimate blood gravities within ±0.002.
6. Apparatus for preparing the copper sulfate solutions: One volumetric flask of 100 ml. capacity and 1 buret, preferably also of 100 ml. capacity, for preparing standard copper sulfate solutions to be stored in 4 oz. bottles. If 2 oz. bottles are used, the flask and buret should be of 50 ml. capacity; if 1 oz. bottles

are used, the flask and buret should be of 25 ml. capacity.

7. Three 4 L. bottles.
8. 1 L. volumetric flask.
9. 500 ml. graduated cylinder.
10. 8 in. funnel and cotton or filter paper.
11. Thermometer, Centigrade, for liquids at room temperature.

Reagents:

1. Oxalate mixture (3:2 ammonium-potassium oxalate), 2%.
   (a) Dissolve 3 gm. ammonium oxalate and 2 gm. potassium oxalate in 250 ml. distilled water. This oxalate mixture, suggested by Heller and Paul, disturbs cell and plasma gravities less than either potassium or ammonium oxalate alone.
2. Crystalline copper sulfate, $CuSO_4 \cdot 5H_2O$. This is preferably purchased in the form of "fine crystals." Otherwise it must be pulverized before using. Four pounds provide a complete set (70) of 100 ml. standard solutions. Ten pounds will probably suffice a laboratory a year.
3. Stock copper sulfate solution of gravity 1.1000. *By weight:* If pure copper sulfate ($CuSO_4 \cdot 5H_2O$) can be weighed, this procedure is simpler than the saturation method.
   (a) Place in a dry 2 L. flask or bottle 170 gm. pure "fine crystals" of copper sulfate, weighed to within 0.1 gm.
   (b) Fill a 1 L. volumetric flask to the mark with distilled water and add, according to the temperature of the water, from a graduated pipet to the liter in the flask, sufficient additional water to bring the volume up to that indicated in the second column of Table 228A. Empty and drain the water from the flask into the bottle with the copper sulfate crystals. Stopper and rotate until all the crystals have dissolved. Then pour the solution once to and from the 1 L. flask to mix the solution within the water left adherent in that flask.
   (c) Or place in a 500 ml. flask or bottle 42.50 gm. copper sulfate, weighed within 0.02 gm.
   (d) Measure with a 250 ml. volumetric flask and a graduated pipet the volume of water indicated in the last column of Table 228A.
   (e) Dissolve and pour the solution back and forth as directed above.

For accuracy in this procedure, it is essential that the copper sulfate shall be pure and have the theoretical 5 molecules of water of crystallization. The crystals can be accurately analyzed by drying at 300°-350° in Pyrex weighing bottles samples of 2 or 3 gm. until the weight is constant, for which 2 or 3 hr. suffice. The theoretical loss of weight for the change from $CuSO_4 \cdot 5H_2O$ to anhydrous $CuSO_4$ is 36.06%. The analysis should be within 0.2% of this. The analyzed copper sulfate must be kept in tightly closed bottles until it is used to make the solution, or it may lose some of the water of crystallization to the air. Even after the 170 or 42.5 gm. portions of pure crystals have been weighed, they must be protected unless used at once; if they lost significant amounts of their crystal water the solution made according to Table 228A would have too little total water, and the gravity would be over 1.1000.

Table 228A. *Volume of water to add to 170 or 42.5 gm. $CuSO_4 \cdot 5H_2O$ to make stock solution of specific gravity 1.100*

Temperature of water (C. °)	*Ml. water to 170 gm. $CuSO_4 \cdot 5H_2O$*	*Ml. water to 42.5 gm. $CuSO_4 \cdot 5H_2O$*
38	1000.2	250.0
36	1001.2	250.3
34	1002.2	250.5
32	1003.2	250.8
30	1004.3	251.1
28	1005.3	251.3
26	1006.3	251.6
24	1007.4	251.8
22	1008.4	252.1
20	1009.5	252.4
18	1010.5	252.6
16	1011.5	252.9
14	1012.6	253.1
12	1013.6	253.4
10	1014.6	253.6

From saturated copper sulfate solution.

4. Stock copper sulfate solution. Prepare 4 L. stock solution. The following directions will yield that amount.
   Saturation:
   (a) Provide 3 dry 4 L. bottles, a Centigrade thermometer for room temperatures, and an 8 in. funnel with a plug of cotton for rapid filtration. Fit the funnel to one of the bottles.
   (b) In another 4 L. bottle place 4 lb. "fine crystals" or pulverized crystalline copper sulfate and 2.5 L. distilled water. (Tap water of specific gravity not over 1.0003, compared with distilled water at the same temperature as 1.0000, may be used.)
   (c) Stopper the bottle and shake vigorously, with repeated inversions, for 5 consecutive minutes by the watch. (It is convenient to have 2 operators who shake during the alternate minutes.)
   (d) The instant the shaking is finished take the temperature to the nearest 0.5°. (It will be 2 or 3° cooler than the water before the saturation because the saturation process absorbs heat.)
   (e) After taking the temperature, immediately decant the solution off the crystals through the funnel with the cotton plug filter, to filter out suspended small crystals.
   (f) Dilute the solution at once to make the stock solution of gravity 1.1000.

Two and one-half liters of saturated solution suffice for more than 4 L. stock solution of gravity 1.100, and this in turn is sufficient for a complete set of 70 standard solutions of 100 ml. vol. each, with enough surplus to provide replacements for the standards that are most used. Smaller or larger amounts of the saturated solution can be made by using proportional amounts of copper sulfate and water.

Dilution:

(a) Measure the volume of saturated solution indicated in Table 228B into a 500 ml. graduated cylinder and pour into a 1 L. volumetric flask.
(b) Allow the upturned cylinder to drain into the flask for 30 sec.
(c) Fill the flask to the mark with water.
(d) Invert several times to mix the solution. The mixing results in a contraction, so that the meniscus now falls below the mark.
(e) Let the flask stand for 1 min. until the solution drains down from the neck.
(f) Add enough additional water to bring the volume to 1 L.

Table 228B. *Ml. saturated copper sulfate solution to be diluted to 1 L. to give the stock solution of specific gravity 1.100*

*Temperature**		*Temperature*		*Temperature*	
C.°	*ml.*	*C.°*	*ml.*	*C.°*	*ml.*
10.0	578	20.0	488	30.0	425
10.5	573	20.5	484	30.5	423
11.0	568	21.0	480	31.0	420
11.5	563	21.5	477	31.5	417
12.0	558	22.0	473	32.0	414
12.5	553	22.5	469	32.5	412
13.0	548	23.0	466	33.0	409
13.5	543	23.5	463	33.5	406
14.0	539	24.0	460	34.0	403
14.5	534	24.5	456	34.5	401
15.0	529	25.0	453	35.0	398
15.5	525	25.5	450	35.5	395
16.0	521	26.0	447	36.0	392
16.5	516	26.5	445	36.5	390
17.0	512	27.0	442	37.0	387
17.5	508	27.5	439	37.5	384
18.0	504	28.0	436	38.0	381
18.5	500	28.5	434	38.5	379
19.0	496	29.0	431	39.0	376
19.5	492	29.5	428	39.5	373
20.0	488	30.0	425	40.0	370

*Temperature in °C. refers to temperature of the saturated solution at the time of saturation (end of shaking for 5 min.)

(g) Mix and pour into a clean, dry, 4 L. bottle. Use the same 1 L. volumetric flask to prepare 3 more liters of the stock copper sulfate solution of gravity 1.100. Each time before the flask is used again, rinse it with water and discard the rinsings.

It is desirable that the saturated solution, the stock solution, and the standard solutions described below all be prepared at within 5° C. of the same temperature. The coefficients of expansion of the saturated and stock copper sulfate solutions are slightly greater than that of water, so that if, for example, the saturated and stock solutions were prepared at 35° C. and the standard solutions at 20°, the standard solutions would have more copper sulfate than intended, enough to make their gravities about 0.001 too high.

5. Standard copper sulfate solutions. In this method 4 oz. bottles are used to hold the standards.

Standard of 1.075 gravity:

(a) Measure 74.3 ml. stock solution of gravity 1.100 from a buret into a 100 ml. volumetric flask.
(b) Dilute to 100 ml. with distilled water and mix.
(c) Transfer to a labeled 4 oz. bottle and stopper to prevent evaporation.

Standard of gravity 1.074:

(a) Rinse the 100 ml. volumetric flask once with water and refill the buret from a 250 ml. Erlenmeyer flask containing the stock solution.
(b) Measure 73.3 ml. stock solution into the volumetric flask and dilute to 100 ml. with distilled water.

Standards of other specific gravities:

(a) Carry out the same procedure for preparation of the entire series down to 1.015, which covers the extreme ranges for blood and plasma. Use Table 228C to determine the amount of stock solution of gravity 1.100 to use.

Procedure for gravity analysis of blood and plasma: Do not apply the tourniquet for more than 1 min. when drawing whole blood. If desired, drops

Table 228C. *Ml. stock copper sulfate solution of specific gravity 1.100 to be diluted to 100 ml. to prepare standard specific gravity solutions**

Gravity	*Ml.*	*Gravity*	*Ml.*	*Gravity*	*Ml.*	*Gravity*	*Ml.*
1.075	74.30	1.060	59.00	1.044	43.00	1.029	28.06
1.074	73.30	1.059	58.00	1.043	42.00	1.028	27.08
1.073	72.20	1.058	57.00	1.042	41.00	1.027	26.10
1.072	71.20	1.057	56.00	1.041	40.00	1.026	25.12
1.071	70.20	1.056	55.00	1.040	39.00	1.025	24.14
1.070	69.10	1.055	54.00	1.039	38.00	1.024	23.15
1.069	68.10	1.054	53.00	1.038	37.00	1.023	22.17
1.068	67.00	1.053	52.00	1.037	36.00	1.022	21.19
1.067	66.00	1.052	51.00	1.036	35.00	1.021	20.20
1.066	65.00	1.051	50.00	1.035	34.00	1.020	19.21
1.065	64.00	1.050	49.00	1.034	33.00	1.019	18.22
1.064	63.00	1.049	48.00	1.033	32.00	1.018	17.23
1.063	62.00	1.048	47.00	1.032	31.00	1.017	16.24
1.062	61.00	1.046	45.00	1.031	30.00	1.016	15.25
1.061	60.00	1.045	44.00	1.030	29.04	1.015	14.26

*Plasma range—1.015-1.035.
Whole blood range—1.035-1.075.

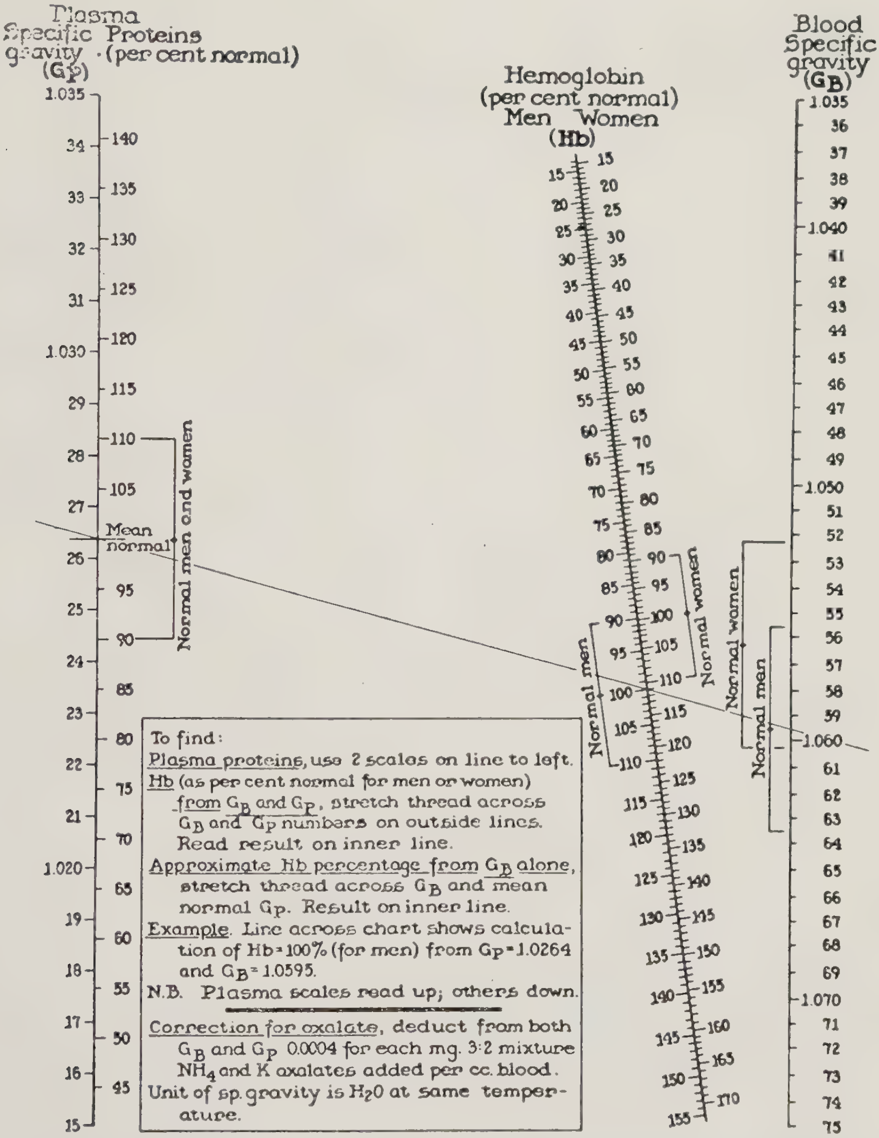
of whole blood may be delivered directly into the standards from the syringe and needle. Or finger blood may be used. Prepare the finger puncture and take the blood up into a capillary tube. If the blood is transferred to a test tube containing anticoagulant, the cells and plasma must be thoroughly mixed immediately before a sample is drawn into a medicine dropper for the gravity test. For this purpose, (1) invert the tube 10 times or (2) raise and lower a glass rod with a mushroom end through the blood 10 times, just before the sample is drawn into the dropper.

Plasma gravity is best determined on plasma from blood that is treated with heparin, 0.2 mg./ml. blood as anticoagulant, since the heparin exerts no measurable effect on the gravity results. Or the Heller-Paul mixture of 3 parts of ammonium oxalate and 2 parts potassium oxalate may also be used for preparation of the blood. **Sodium citrate must not be used.**

Serum may be used in place of plasma. The gravity of serum indicates its protein content without the 0.2 or more gm. fibrinogen/100 ml. removed by the clotting.

Deliver the drop of serum, plasma, or whole blood from a height of about 1 cm. above the solution from a medicine dropper, syringe needle, or capillary tube. When the drop is delivered, steady the dropper on the edge of the bottle.

The delivered drop breaks through the surface



To find:

Plasma proteins, use 2 scales on line to left.

Hb (as per cent normal for men or women) from G_B and G_P, stretch thread across G_B and G_P numbers on outside lines. Read result on inner line.

Approximate Hb percentage from G_B alone, stretch thread across G_B and mean normal G_P. Result on inner line.

Example. Line across chart shows calculation of Hb=100% (for men) from G_P=1.0264 and G_B=1.0595.

N.B. Plasma scales read up; others down.

Correction for oxalate, deduct from both G_B and G_P 0.0004 for each mg. 3:2 mixture NH_4 and K oxalates added per cc. blood.

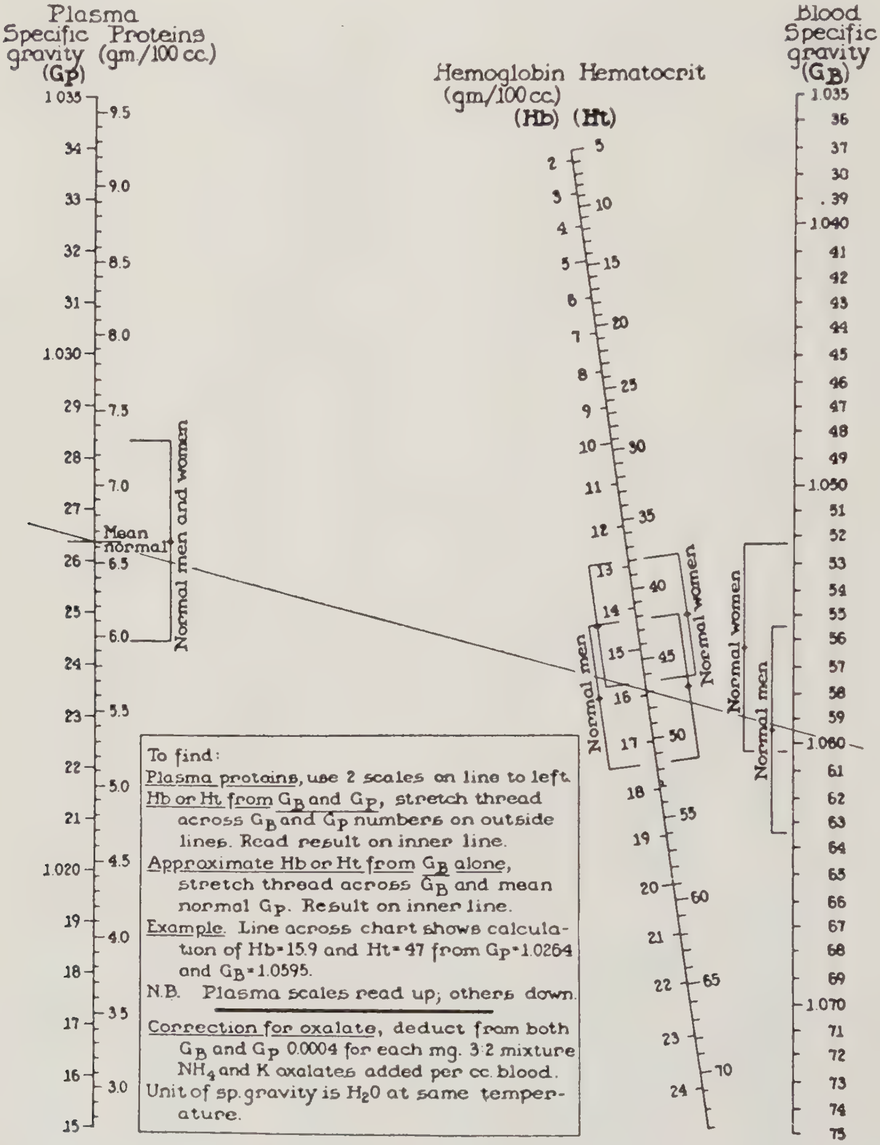
Unit of sp. gravity is H_2O at same temperature.

Fig. 360. Line chart for calculating percentages of normal plasma proteins and hemoglobin from gravities of plasma and blood. (Courtesy U. S. Navy Research Unit, Hospital of the Rockefeller Institute for Medical Research, New York City, by permission of the Surgeon General, U. S. Navy; from Simmons, J. S., and Gentzkow, C. J., editors: Laboratory methods of the United States Army, ed. 5, Philadelphia, 1944, Lea & Febiger.)

film of the solution and then penetrates 2-3 cm. below the surface; within 5 sec. the momentum of the fall is lost and the drop then begins to rise, becomes stationary, or continues to fall. The gravity of the drop relative to the solution does not change appreciably until the drop has been immersed in the solution for another 10-15 sec., and there is ample time to note its behavior during this interval. **If the drop is lighter** than the test solution, it will rise, perhaps only a few mm., and may begin to sink immediately afterward. **If the drop is of the same gravity** as the standard test solution, it will become stationary for this interval and then fall. **If the drop is heavier,** it will continue to fall during this interval. It is to be emphasized that the behavior during the 10 sec. after the drop has lost momentum of its fall into the solution indicates whether the drop is lighter or heavier than the test solution—if it rises at all during this period, it is lighter than the standard.

For the test add a drop of the blood, serum, or plasma to various standards and select as the reading the standard that is of the same specific gravity as the blood, i.e., where the drop neither rises nor falls but remains stationary.

Calculation: Calculations are made with lined charts that take into account plasma protein concentration, hemoglobin concentration in blood, and



To find:

Plasma proteins, use 2 scales on line to left.

Hb or Ht from G_B and G_P, stretch thread across G_B and G_P numbers on outside lines. Read result on inner line.

Approximate Hb or Ht from G_B alone, stretch thread across G_B and mean normal G_P. Result on inner line.

Example. Line across chart shows calculation of Hb=15.9 and Ht=47 from Gp=1.0264 and G_B=1.0595.

N.B. Plasma scales read up; others down.

Correction for oxalate, deduct from both G_B and G_P 0.0004 for each mg. 3:2 mixture NH_4 and K oxalates added per cc. blood.

Unit of sp. gravity is H_2O at same temperature.

Fig. 361. Line chart for calculating plasma proteins, hemoglobin, and hematocrit from gravities of plasma and blood. (Courtesy U. S. Navy Research Unit, Hospital of the Rockefeller Institute for Medical Research, New York City, by permission of the Surgeon General, U. S. Navy; from Simmons, J. S., and Gentzkow, C. J., editors: Laboratory methods of the United States Army, ed. 5, Philadelphia, 1944, Lea & Febiger.)

hematocrit percentages. Hemoglobin and hematocrit values are estimated by laying a straightedge across the charts. See Figs. 360 and 361.

Accuracy of method: The hemoglobin content by the gravity method has been found regularly within 2% of the correct value measured by oxygen capacity. Usually the accuracy is within ±0.2 gm. In pathologic blood conditions, such as pernicious anemia, the error can be larger.

This method has been used widely in official circles and has been definitely accepted.

METHODS FOR STANDARDIZATION OF ROUTINE CLINICAL HEMOGLOBINOMETRY

The following 2 methods can be used for standardization of the routine methods discussed in the preceding pages.

Methods of hemoglobin determinations based on gasometric analysis

These determinations are performed in the Van Slyke-Neill apparatus. The methods were originally based on the oxygen capacity of blood. Various refinements, including the determination of carbon monoxide of the blood, have been added to the original method.[96] The method has a high degree of accuracy, but the procedure is too cumbersome for routine testing.

Even with this highly refined method, there remain some questions concerning normal values of human hemoglobin. In the past, it was assumed that the constant for oxygen combining with each gm. hemoglobin (**Hüfner factor**) was 1.34 vol.%. These data were based on observations on ox and horse blood.

$$\text{Gm. Hb/100 ml. blood} = \frac{20.9}{1.34} = 15.58$$

Each gm. oxyhemoglobin combines with 1.34 vol.% oxygen. In order to determine the number of gm. hemoglobin in 100 ml. blood, the oxygen capacity of blood is determined, and the vol.% O_2 in 100 ml. blood is divided by 1.34. The oxygen-binding capacity of 100 ml. blood is normally 20.9 vol.%.

Oxygen capacity measures only oxyhemoglobin and does not include hemoglobins which do not combine with oxygen, for instance, COHb, methemoglobin, and sulfhemoglobin.

Determination of oxygen content and capacity

A macromethod that makes use of the Van Slyke manometric apparatus and a micromethod using the Natelson microgasometer are described. The design and general instructions for the use of the instruments are given in the clinical chemistry section of this text and will not be repeated here.

The principle of the methods is identical. Blood is reacted with ferricyanide which oxidizes the hemoglobin to methemoglobin. This reaction is carried out in a partial vacuum in the chamber of the apparatus and results in the liberation of the oxygen which was combined with the hemoglobin and of gases dissolved in blood. The pressure of the gases liberated is measured and recorded; then an alkaline hyposulfite reagent which absorbs the oxygen is introduced into the chamber. A second pressure measurement is made, and the difference in these 2 pressure measurements then represents the pressure of the oxygen liberated.

This procedure differs from many described in that an alkaline rather than acid ferricyanide reagent is used which results in the liberation of oxygen quantitatively without the liberation of any carbon dioxide.

Reagents:

1. Ferricyanide-borate-saponin solution.

   Dissolve 1.6 gm. potassium ferricyanide, 0.5 gm. saponin, and 3 gm. sodium tetraborate decahydrate in distilled water and dilute to a total volume of 100 ml.

When used in the macroprocedure, this reagent is deaerated in the apparatus as part of the procedure, but when used in the microprocedure, it must be deaerated before introduction into the chamber. This may be accomplished very simply by placing the reagent in a bottle, covering it with a layer of mineral oil, and evacuating the bottle with a water aspirator or a vacuum pump. The layer of oil permits the escape of air bubbles from the solution but limits the diffusion of air back into it. This reagent is stable for approximately 2 wk. and in contrast to other reagents used in microgasometer technics should not be stored in contact with mercury.

2. Alkaline hyposulfite reagent.

   Place 2 gm. sodium hyposulfite ($Na_2S_2O_2$) in a small bottle or vial. Add 10 ml. 2N sodium hydroxide. Quickly dissolve the salt and cover with a layer of mineral oil. Deaerate as described for the ferricyanide reagent. This reagent is best prepared on the day of its use.

3. Caprylic alcohol.

Collection of sample: The sample is heparinized blood. When the oxygen content is to be determined, arterial blood is preferable since the results are more readily interpretable. Capillary blood, which is largely arterial, may be substituted, but usually yields values approximately 5% lower than arterial blood. There may be instances in which results obtained with capillary blood are not strictly comparable to those obtained with arterial blood. Blood for oxygen content must be drawn in such a way that exposure to air is excluded, and prior to analysis it should be stored under a layer of mineral oil. If only oxygen capacity is to be determined, then the source of blood is immaterial, and obviously the precautions concerning exposure to air do not apply.

1. Draw blood into a syringe which has been wet with a solution of heparin.
2. Plug the needle with a rubber stopper and mix the blood by rotation of the syringe.
3. Remove the stopper and express a few drops of blood from the needle.
4. Transfer the blood to a tube under a layer of oil. Leave sufficient blood in the syringe and transfer this to a flask for aeration for the oxygen capacity determination.
5. Aeration of blood for the capacity measurement is easily accomplished by placing the blood in a flask, where it will be spread out in a thin layer.
6. Shake for 5-10 min.

Capillary blood should be collected in heparinized tubes of about 1.5 mm. bore. A sample of about 0.05 ml. content can be transferred to a small tube under oil. The sample for oxygen capacity can be transferred to the bottom of a tube of about 10 mm. diameter and aerated by flicking the tube for about 3 min.

Procedure for Van Slyke apparatus

For 2 ml. blood:

1. Place 10 ml. ferricyanide reagent in the cup above the chamber and run into the chamber.
2. Draw 2 drops caprylic alcohol into the chamber, seal the top stopcock, and draw the level of mercury down just into the arm below the the chamber in order to minimize the surface of mercury in contact with the reagent.
3. Shake 3 min. to extract the air in the reagent.
4. Eject the extracted air from the chamber and force 6 ml. ferricyanide reagent up into the cup.
5. As quickly as possible after the solution has been run up into the cup, run the blood sample into the chamber of the apparatus. It is convenient for this purpose to use pipets which are graduated between the marks and which are fitted with a rubber tip.
6. Run 1 ml. reagent in the cup into the chamber after the blood sample, leaving 5 ml. in the cup.
7. Seal the top stopcock with mercury, aspirate the excess reagent from the cup, and rinse the cup with distilled water.
8. Again draw the mercury level down just below the chamber and extract the oxygen in the solution by shaking 3 min.
9. When the extraction is completed, bring the meniscus of the solution in the chamber to the 2 ml. mark and read p_1 from the manometer. This pressure is the resultant of the oxygen and nitrogen extracted from the blood.
10. To absorb the oxygen, lower the level of liquid in the chamber until the vol. of the space above it is approximately 5 ml., then close the stopcock to the mercury bulb.
11. Add 1.5 ml. air-free hyposulfite solution to the cup and admit this reagent to the chamber 1 drop at a time. As the reagent is added, it absorbs oxygen, and the level of mercury in the manometer falls. After the addition of several drops of reagent, no further perceptible fall of mercury in the manometer will occur.
12. When this point is reached, open the stopcock to the mercury bulb and permit the level of the solution to flow up in the chamber until it stops. The mercury bulb should be in its lower position when this operation is carried out. Now permit the remainder of the hyposulfite reagent to flow into the chamber and seal the top stopcock with mercury.
13. Allow a total of at least 4 min. to elapse after the first addition of hyposulfite, then bring the meniscus of the solution to the 2 ml. mark, and read P_2 from the manometer.
14. The oxygen pressure pO_2 is $p_1 - p_2$.
15. Record the temperature in the jacket of the extraction chamber.
16. Multiply the pO_2 by the proper factor from Table 229 to calculate the oxygen content in vol.% or mM/L.

Table 229. *Factors for calculation of O_2 content of blood (Van Slyke apparatus)*

Temperature	*Vol.% O_2 in blood sample*		*mM/L. O_2 in blood sample*	
	1 ml.	*2 ml.*	*1 ml.*	*2 ml.*
15	0.2493	0.1251	0.1113	0.0558
16	0.2485	0.1246	0.1109	0.0556
17	0.2478	0.1242	0.1105	0.0554
18	0.2468	0.1237	0.1101	0.0552
19	0.2459	0.1232	0.1097	0.0550
20	0.2450	0.1228	0.1093	0.0548
21	0.2441	0.1224	0.1089	0.0546
22	0.2432	0.1219	0.1085	0.0544
23	0.2423	0.1215	0.1081	0.0542
24	0.2414	0.1210	0.1077	0.0540
25	0.2406	0.1206	0.1074	0.0538
26	0.2398	0.1202	0.1070	0.0536
27	0.2390	0.1198	0.1067	0.0534
28	0.2382	0.1193	0.1063	0.0532
29	0.2374	0.1189	0.1059	0.0530
30	0.2366	0.1185	0.1055	0.0529
31	0.2358	0.1181	0.1052	0.0527
32	0.2350	0.1177	0.1048	0.0525
33	0.2342	0.1173	0.1044	0.0524
34	0.2333	0.1169	0.1041	0.0522

For 1 ml. blood: The technic is the same as described for 2 ml. except that less ferricyanide is used; 7.5 ml. of the ferricyanide solution are first freed of air by 3 min. shaking, then 6 ml. are returned to the cup, and 1 ml. is returned with the blood sample to the chamber so that the final mixture is 1 ml. blood plus 2.5 ml. ferricyanide reagent.

Note: The oxygen content as determined represents both the oxygen bound to hemoglobin and the oxygen which is physically dissolved in the blood. In circulating arterial blood the correction for dissolved oxygen is quite small and approximates 0.25 vol.%.

However, when blood is saturated with air for the determination of its oxygen capacity, then the amount of oxygen dissolved varies with the temperature and with atmospheric pressure and reaches significant levels. When the oxygen capacity measurement is used to calculate the hemoglobin concentration, it then becomes imperative that the oxygen as determined be corrected for this dissolved oxygen. When blood is saturated with air at 25° C. and 760 mm. pressure, the physically dissolved oxygen is 0.62 vol.%. This value changes with atmospheric pressure to only a small extent, the magnitude of change being approximately 0.02 vol.% for each 20 mm. change in pressure; the dissolved oxygen increases with increasing pressure and vice versa. The dissolved oxygen increases as the temperature falls and vice versa; the magnitude of this change is approximately 0.017 vol.% per degree.

To calculate the hemoglobin concentration of a blood sample, the oxygen content in vol.% air-saturated blood is corrected for the dissolved oxygen, and the corrected value is multiplied by 0.746 to give gm.% hemoglobin. This factor is derived from the observations that 1 gm. adult human hemoglobin combines with 1.34 ml. oxygen (1 gm.% Hb = [oxygen capacity in vol.% − dissolved oxygen in vol.%] × 0.746).

Another important parameter which can be calculated from the determination of both the oxygen content and capacity of a blood sample is its degree of saturation with oxygen. The **oxygen saturation** is calculated as follows:

$$\%\ \text{Oxygen saturation} = 100 \times \frac{\text{Vol.\% } O_2 \text{ bound by Hb in blood as drawn}}{\text{Vol.\% } O_2 \text{ bound by Hb in saturated blood}}$$

Procedure for Natelson microgasometer

Reagents: The reagents are as described for the Van Slyke procedure. Note the precaution with regard to the ferricyanide reagent that it must be deaerated before it is introduced into the chamber and preferably only shortly before its use.

Procedure:

1. Draw up 0.03 ml. blood, followed by a 0.1 ml. mercury plug.
2. Draw up 0.02 ml. caprylic alcohol, 0.03 ml. ferricyanide reagent, 0.02 ml. caprylic alcohol, and 0.03 ml. ferricyanide reagent in that order and follow with mercury until all the reagents are beyond the stopcock.
3. Close the stopcock and retreat with the plunger until all the reagents are drawn into the lower portion of the bulb.
4. Shake for 3 min.
5. Bring the meniscus to the 0.12 ml. mark and take a reading on the manometer.
6. Retreat again until the reagents are in the lower portion of the bulb and shake for 2 min.
7. Advance the meniscus to the 0.12 ml. mark and take a second reading.
8. When 2 consecutive readings agree, all of the oxygen is liberated, and this manometer reading is p_1.
9. Advance the screw so that the mercury in the manometer barely reaches the top and open the stopcock.
10. Advance the screw until a drop of mercury protrudes from the tip of the pipet.
11. Draw up 0.03 ml. hyposulfite reagent and follow with mercury until the reagent is beyond the stopcock.
12. Close the stopcock.
13. Retreat with the plunger until the reagents are in the lower part of the bulb.
14. Shake 3 min. to absorb the oxygen, bring the meniscus of the solution to the 0.12 ml. mark, and read the pressure in the manometer. This is p_2.

Calculations:

Oxygen in vol.% = $(p_1 - p_2)$ × factor from Table 230.

Oxygen in mM/L. = Vol.% × 0.446

Table 230. *Factors for calculating oxygen in volumes per cent**

Temperature	*Factor*	*Temperature*	*Factor*
18	0.494	27	0.478
19	0.492	28	0.476
20	0.490	29	0.475
21	0.488	30	0.473
22	0.486	31	0.472
23	0.485	32	0.470
24	0.483	33	0.468
25	0.481	34	0.467
26	0.480	35	0.465

*From Natelson, S.: Microtechniques of clinical chemistry for the routine laboratory, Springfield, Ill., 1957, Charles C Thomas, Publisher.

Gas chromatography

Gas chromatography allows analysis of whole blood for oxygen, nitrogen, and carbon dioxide contents in just a few minutes. Fisher's clinical gas partitioner* requires 0.1 ml. blood for complete gas analysis and measures content or capacity. Gas chromatography uses the established technic of column chromatography. In gas chromatography an inert carrier gas dilutes the gaseous specimen and carries it through chromatographic columns at different speeds as molecules of different gases interact with the packing material to different degrees. This interaction may consist of adsorption on the packing material. The different components consequently separate and are eluted from the columns at different times.

Methods of hemoglobin determinations based on measurement of iron content of blood

Basically, the many modifications for determining iron content of blood depend upon liberation of the 4 iron molecules attached to the 1 globin molecule in hemoglobin. The Wong method (p. 229) is a typical technic. The subject of iron determination in blood has been very thoroughly covered in a symposium on hemoglobinometry.[97] The iron method is too cumbersome for routine clinical work, but its accuracy (±1%) has made it a popular method for the calibration of clinical methods. If whole blood is used for the iron determination, the result must be corrected for the plasma iron. One gram hemoglobin contains 0.335 mg. iron.

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*Fisher Scientific Co., New York, N. Y.

CRITIQUE OF HEMOGLOBINOMETRY

Determination of hemoglobin is perhaps one of the most common laboratory procedures in a hematologic study of patients; yet there exists more inaccuracy in this determination than in many other tests. The subject was of sufficient interest to warrant its presentation before the American Society of Clinical Pathologists and the College of American Pathologists in 1950. As a result, a most comprehensive monograph on clinical hemoglobinometry was published.[97] These authors stated: "Recent surveys have revealed wide discrepancies in hemoglobin measurements in different clinical laboratories. These are in part due to errors in measurements, differences in methodology, the uncertainties encountered with the continuance of obsolete color methods that use artificial standards, and, perhaps most important, because of a lack of an accepted standard of reference for hemoglobinometry."*

Karr and Clark[98] call attention to inaccurately calibrated pipets, both for blood sampling and for measuring diluents, as a source of error. They also include careless technic as an important factor in poor results. For accurate hemoglobin determination, all equipment used must be calibrated and standardized. If it is impossible to calibrate instruments because of lack of properly trained personnel, the work can be done either by experts in the vicinity or by supply houses which furnish such equipment. Once a photoelectric colorimeter has been calibrated, it should be checked from time to time for accuracy.

Regarding methodology, today we are solving one of the problems that has plagued us in hemoglobinometry for the past 80 yr. by the use of photoelectric colorimetry. Obviously such precision instruments have a considerable advantage over all methods utilizing visual colorimetry. However, the use of a precision instrument does not guarantee precise results unless all technical aspects in the use of the instruments are properly understood and carried out.

It would be exceedingly helpful if there could be some universal agreement as to the normal values for hemoglobin. Here in the United States, it is generally agreed that the average normal hemoglobin concentration for males is 15.8 gm./100 ml. blood, and for females, 13.9.[99] Since these figures represent accumulations of great numbers of observations, it is suggested that this be used as a standard for normals in the United States. From a clinical standpoint, it is important to remember that there are ranges of hemoglobin values that are still within the normal range for both males and females. Such ranges as given by Albritton are for adult males 14-18 gm./100 ml. blood and for adult females 11.5-16 gm./100 ml. Acceptance of these ranges of normal values must not be construed as placing a seal of approval on poor technic which results in variations perhaps on the same sample of blood.

The time-honored tradition of reporting values in percentage is so well established that it has been difficult in many places to discard this method of reporting. The time has now come when **reporting hemoglobin in percentages should be discontinued.** A most casual perusal of the various "normals" for 100% for the different instruments used in hemoglobinometry reveals great disparity between the number of gm. hemoglobin/100 ml. that equals 100%. In Table 231 are figures indicating some of the values considered 100% in various methods.

Table 231

Instrument	*Gm. Hb/100 ml. blood*
Sahli	13, 8, 14, 14.5, 17, 17.2, 17.3, etc.
Sahli-Leitz	15
Dare	13.75, 13.77, 13.8, 15.9, 16.9
Tallqvist	13.8, 15.6, 15.8, 16.25, 16.5
Newcomer	16.92
Haldane	13.8
Haden-Hausser	15.4, 15.6, 16.7
von Fleischl-Miescher	15.8
Van Slyke	16.9

To convert from % hemoglobin to gm. hemoglobin in 100 ml. blood, multiply the gm. standard of the apparatus used by the % hemoglobin as read on the hemoglobin scale.

To convert from gm. hemoglobin/100 ml. blood to % hemoglobin, divide the number of gm. in the patient's blood by the gm. standard of the apparatus and multiply by 100.

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*From Sunderman, F. W., et al.: Am. J. Clin. Path. **23**:519, 1953.

The ultimate choice for hemoglobin determination should be related to the purpose for which determinations are made, with appreciation for the limits of accuracy of the method to be used. Generally speaking, the less expensive types of equipment with relatively simple technics might be chosen in situations in which screening is all that is desired. The Sahli, Haden-Hausser, Dare, and Spencer instruments fit into this category. These methods have shown errors ranging from ±5 to 40%, but "with a close check on the standards, it may be possible with careful technic to hold the error to ±5 to 10%."[97]

In clinical laboratories and hospitals and even in private offices of physicians, where a fair amount of hematology is performed daily, the cost of a well-standardized photoelectric colorimeter is not so prohibitive as to preclude its use routinely. In modest-sized hospital laboratories and in physicians' offices, it is recommended that the photoelectric colorimeter be of such construction as to permit its use for multiple determinations as well as of hemoglobin. Purchase of a photoelectric colorimeter which has been standardized for hemoglobin determinations only and which has the convenience of reading the concentration of hemoglobin directly from the ammeter on the instrument is not economical in the smaller laboratory. In large laboratories where several photoelectric colorimeters are available and where the use of one could be limited to determinations of hemoglobin, perhaps there may be some justification for the purchase of an instrument calibrated for hemoglobin only. Regardless of which type of photoelectric instrument is purchased, attention is again called to the fact that a **paramount prerequisite of its use is accurate calibration.** The fact that a hemoglobin determination has been performed with a photoelectric apparatus is no guarantee that it is accurate. This precision is directly proportional to the accuracy of calibration of the instrument, as well as to precision in the performance of the technic.

One satisfactory method for the clinical laboratory using photoelectric colorimetry is the Sheard-Sanford technic or some modification of it in which oxyhemoglobin is measured. Accuracy in this method is somewhere in the range of ±4%. Sunderman et al. stated: "It is the considered opinion of members of this panel that for routine clinical use, the carboxyhemoglobin methods, the cyanmethemoglobin methods, and the oxyhemoglobin colorimetric methods (in the order named) are the most reliable and trustworthy."* The use of poisonous cyanide reagents in the cyanmethemoglobin method is a major objection to this method. Our objection to the carbon monoxyhemoglobin methods is the introduction of a complicating element (a tank of carbon monoxide or a source of illuminating gas) that is hardly justified for routine clinical procedures. This choice of oxyhemoglobin method is made with a full realization that it does not measure hemoglobin derivatives such as carboxyhemoglobin, methemoglobin, and sulfhemoglobin.

The gasometric methods are obviously unsuited for clinical hemoglobinometry, requiring special equipment and being time-consuming. The precision of such methods favors their use in the calibration of photoelectric instruments. However, many prefer methods based on iron analyses (accuracy ±1%) for calibration. These methods are likewise too cumbersome for routine clinical work.

In the calculation of erythrocyte constants (mean corpuscular volume, mean corpuscular hemoglobin, and mean corpuscular hemoglobin concentration), the hemoglobin determinations must be based on methods having a high degree of accuracy.

DETERMINATION OF ABNORMAL HEMOGLOBINS BY ELECTROPHORESIS

Abnormal hemoglobins may be demonstrated in the Tiselius apparatus. This equipment is expensive, and procedures are laborious. A substitute for this apparatus has been found in the form of paper electrophoresis outfits.

The basic principle of the method is the observation of the migration of a drop of protein (stained or unstained) on a piece of filter paper moistened with buffer solution in an electrophoresis hookup.[100-103]

The various hemoglobins detected by electrophoresis are known as hemoglobins A (normal adult), F (fetal), C, D, E, G, H, I, and S (sickle cell). Descriptions of the significance of these varieties of hemoglobin may be found in the discussion of hemoglobinopathies. The relative mobilities of these hemoglobins have the following order: A, F, E, S, D, and C. The apparatus is described in the discussion of electrophoresis.

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*From Sunderman, F. W., et al.: Am. J. Clin. Path. 23:519, 1953.

ALKALI DENATURATION TEST FOR FETAL HEMOGLOBIN (HEMOGLOBIN-F)*

Material:

1. 2 to 4 ml. of oxalated venous blood.
2. N/12 Potassium hydroxide.
   Dilute 1 part N/4 potassium hydroxide solution with 2 parts distilled water. Keep the stock solution in a refrigerator in tightly stoppered paraffin-lined bottle.
3. Precipitating reagent.
   Add 400 ml. distilled water and 2 ml. concentrated hydrochloric acid (10N) to 400 ml. saturated solution of ammonium sulfate.
4. Conical graduated centrifuge tubes.
5. Toluene, c.p.
6. Physiologic saline, 0.85%.
7. Stopwatch.
8. Photoelectric colorimeter.

Procedure:

1. Place the blood in a 15 ml. conical centrifuge tube.
2. Wash once with saline solution, centrifuge, and discard washings.
3. Add 1.5 vol. distilled water and 0.4 vol. toluene.
4. Stopper and shake vigorously for 2-3 min. and allow to stand overnight in a refrigerator at about 4°-10° C. If it is desirable to proceed immediately, then shake for 5-6 min. and continue without refrigeration.
5. Centrifuge for 20 min. at 2000-5000 r.p.m.
6. Aspirate and discard upper clear layer and middle layer of sediment.
7. Filter the hemoglobin solution remaining in the centrifuge tube. The concentration of this hemoglobin solution will be between 8 and 12 gm.% and need not be checked.
8. Transfer 1.6 ml. N/12 potassium hydroxide solution to a Kahn tube and keep in a 20° C. water bath for several min.
9. Add 0.1 ml. test solution of hemoglobin to the precipitating reagent, rinsing the pipet 6 times and agitating the tube gently 10-20 times. As soon as the hemoglobin solution has been added, time with the stopwatch.
10. At the end of exactly 1 min., add 3.4 ml. precipitating reagent of ammonium sulfate.
11. Stopper, invert 3-4 times, and filter into a clean tube (**solution A**).
12. Determine the hemoglobin concentration of solution A in a photoelectric colorimeter.
13. Prepare a 1:200 dilution original hemoglobin solution by adding 0.02 ml. (with a Sahli pipet) to 4 ml. distilled water (**solution B**).
14. Determine the hemoglobin concentration of solution B in a photoelectric colorimeter.

Calculations:

$$\%\ \text{fetal hemoglobin} = \frac{\text{Hb concentration sol. A} \times \frac{1}{4}}{\text{Hb. concentration sol. B}} \times 100$$

The factor ¼ is used to compensate for differences in dilutions of solutions A and B, which are 1:50 and 1:200, respectively.

Normal (hemoglobin-F):

Newborn infants	55-85%
12 to 18 mo.	0-10%
1½ to 5 yr.	0- 3%
Over 5 yr.	0- 1.8%

Comments: For routine clinical studies, visual observation only of solution A may suffice since concentrations of 2% or more of hemoglobin-F will impart a brownish or red color to the filtrate. Concentrations below 2% hemoglobin-F result in colorless solutions. Diagnostically, values above 2% are significant and therefore this qualitative determination alone may be all the procedure necessary.

Attention is directed to the high concentrations (55-85%) of hemoglobin-F in newborn infants and the existence of elevated concentrations of this hemoglobin component generally up to 2 yr. of age. Occasionally, children up to 5 yr. of age may show slightly increased values (up to 3 or 4%). Allowance should be made for these ranges in the study of disease under these conditions.

Elevations of alkali-resistant hemoglobin-F occur almost constantly in the hereditary hemolytic syndromes. In thalassemia, concentrations up to 90% hemoglobin-F have been found, whereas in sickle cell anemia the concentration may reach 25%. In hereditary spherocytosis, levels up to 20% hemoglobin-F are not unusual. The levels reached in a particular patient do not necessarily distinguish these various syndromes from one another.

The finding of moderate increases of fetal hemoglobin is not necessarily diagnostic of familial or hereditary hemolytic disease. Values up to 5 or 10% have been found in chronic leukemia, multiple myeloma, and pernicious anemia. In almost all other anemias, such as iron-deficiency anemia and acquired hemolytic anemias, hemoglobin-F concentrations are normal.

VOLUME PER CENT OF ERYTHROCYTES (CELL VOLUME PER CENT OR HEMATOCRIT)

Measurement of the volume of packed red blood cells/100 ml. blood by the hematocrit technic is the most accurate and simplest method of determining the presence or absence of anemia or polycythemia and of measuring their degree. In comparison, hemoglobin determination is less accurate, and red blood cell counting is far less accurate.

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*Submitted by Dr. A. I. Chernoff as modification technic of Singer et al.[104]

Relative to these determinations Wintrobe,[105] in a personal communication, stated: "It has been our practice for years to do just this and to reserve the determination of hemoglobin and red cell counts for those instances where one wishes to know what the average size and hemoglobin content of the red cells are. When this is needed, then the hemoglobin should be measured carefully, and the red cell counts should be carried out meticulously. Even then, red cell counting carries a substantial margin of error. Certainly, as red cell counting is done in ordinary practice, it carries an error of anywhere from 20 to 50% or more. I see many instances of erroneous blood cell counting and, strangely enough, most physicians are unaware of the large margin of error in this determination."*

The hematocrit determination made on venous blood is called venous hematocrit. It measures the relative amount of red cells in the plasma of the blood and thus measures the concentration of red blood cells. It does not measure the total blood volume. To determine the latter, other methods should be used or allowance should be made for the plasma trapped in the erythrocyte column. The latter can be measured with radioiodinated albumin or determined from a graph based on standardized methods of centrifugation. The total body hematocrit is roughly 9% higher than the venous hematocrit.

The hematocrit values form the basis of some of the blood constants and indices, which serve to characterize types of anemias.

In preoperative and postoperative hemorrhage, hematocrit examinations are useful in controlling blood replacement.

As just mentioned, the hematocrit technic is a useful method to determine the presence or absence of anemia or of polycythemia.

HEMATOCRIT[106]

Normal:

Adult male: average, 47 vol.%; range, 40-54.
Adult female: average, 42 vol.%; range, 37-47.

Macromethod for hematocrit determination—Wintrobe

Equipment:

1. Wintrobe hematocrit tube.

   This is a heavy cylindrical tube marked off on the exterior on the left-hand column from 0-10 cm. and on the right from 10-0, beginning at the top of the tube. Each main division represents 1 cm. and each small division 1 mm. To convert the reading to percentage when the tube has been filled to the top, multiply the figure in cm. by 10 or read directly in mm.

   Collect blood by venipuncture, place in 3:2 oxalate mixture (p. 1100), and mix thoroughly but gently.
2. Hematocrit-filling pipet.

   These are long glass capillary pipets or preferably metal cannulae which fit inside the Wintrobe tube. Disposable long pipets are especially useful for this purpose.
3. Centrifuge.

   This should be capable of a speed of 3000 r.p.m. or 2000 g. The type of centrifuge is important since a standardized reproducible method requires a constant centrifugal force which in turn depends on the radius and the speed of the centrifuge and the direction of the centrifugation. In the routine clinical laboratory, absolute packing of the red cells usually cannot be achieved since a centrifugal force of about 200,000 g is required. The test is reproducible if a standard time and a standard centrifugal force are used.

Procedure:

1. Mix the oxalated venous blood thoroughly.
2. Draw up sufficient blood to fill a Wintrobe tube, slightly more than 1 ml. in a dry filling pipet.
3. Insert the tip of the pipet to the bottom of a clean dry Wintrobe hematocrit tube. By gentle pressure of the rubber bulb at the end of the pipet, fill the hematocrit tube, slowly pulling up on the pipet. Avoid introducing air bubbles. The tube should be filled to exactly the **0** on the left side of the tube. If the tube is filled beyond this point, remove enough blood so that the meniscus is at exactly **0** point. If insufficient blood is available for filling the entire hematocrit tube, the tube may be filled to any mark and the vol.% packed erythrocytes calculated by dividing the volume of packed erythrocytes by the total volume of blood in the tube. If the tube is filled to exactly to **0** mark, readings can be taken directly from the tube.
4. Close the open end of the tube with a small rubber stopper supplied with the apparatus to prevent evaporation. This is not necessary if the tube has been filled to the **0** mark. If less than this amount of blood is used, the open end of the tube must be closed to prevent evaporation during centrifugation.
5. At this point if desired, the Wintrobe tube may be placed in a rack or in an upright position and allowed to stand for 1 hr. to determine the sedimentation rate and then centrifuged for the hematocrit determination. If the sedimentation rate is not desired, centrifuge for 30 min. at 3000 r.p.m.
6. Read the volume of packed red blood cells directly on the tube at the meniscus of the erythrocyte column. Read from the bottom up and multiply the reading on the right side (cm.) of the tube by 10 to obtain the vol.% of packed erythrocytes.

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*From Wintrobe, A. A.: Personal communication.

Examine the hematocrit tube for other features. Beginning at the top of the tube, observe the **fatty layer,** which is normally barely visible. When the blood fat content is exceedingly high, a layer of several ml. may be seen at the top of the tube. The main column in the tube from the top down is plasma. Normally, this **plasma layer** is a pale straw-colored yellow and is fairly clear. A cherry-red color is evidence of excessive hemolysis. Hemolysis may be due to faulty technic, such as collection of blood in wet syringes or wet needles or placing it in a wet hematocrit tube. Hemolysis may also occur as a result of exposure of the blood to high temperatures (radiators) before the determination is made or as a result of some inherent disease of the red blood cells. In the presence of excessive hemolysis, the hematocrit values will be abnormally low and, therefore, incorrect. In such cases, a new blood specimen should be obtained, and all precautions should be taken to prevent hemolysis.

The plasma may also be tinted a deep yellow or almost brown color, indicating the presence of jaundice. The intensity of this color is an indication of the intensity of the jaundice.

Just beneath the plasma layer is a reddish gray layer or **buffy coat,** which normally varies in thickness from 0.5-1 mm. This layer consists of packed leukocytes and platelets, the thickness of the layer roughly representing the total counts of leukocytes and platelets. If this layer is exceedingly thin, e.g., less than 0.5 mm., it may indicate a reduction in platelets or in leukocytes. If it is greater than 1 mm., it usually represents an increase in the leukocyte count above 10,000/c.mm. Sometimes this layer may subdivide into 2 indistinctly separated layers, of which the uppermost portion is rich in platelets and the lower almost exclusively leukocytes. In marked leukopenia, the leukocyte layer may be aspirated and spreads made for differential counting in order to obtain a greater concentration of white cells. This is not recommended as a routine procedure because white cells collected in any anticoagulant show morphologic changes sufficiently great to give a wrong impression of the true character of these cells.

In searching for **lupus erythematosus (L.E.) cells,** the buffy coat of the hematocrit tube serves as a very useful concentration method.

Permit the blood specimen to remain at room temperature for approximately 2 hr. before centrifugation if the preparation is to be examined for L.E. cells.

Some workers who collect **bone marrow** preparations in an anticoagulant use the hematocrit tube method as a rough estimate of the relative cellularity of the bone marrow. Various figures are given for the normal thickness of the buffy coat of bone marrow, ranging from 5-10 mm. This crude method is not a true gauge of the cellularity of the bone marrow, varying considerably with the technic of the operator who performs the aspiration and with the amount of bone marrow removed.

In the event that less than 1 ml. blood is available, the hematocrit determination made with this lesser quantity is calculated as follows.

Suppose that the hematocrit tube has been filled to the 6 mark (60 mm.). The vol. packed red cells after 30 min. centrifugation is at the 2.4 mark (24 mm.).

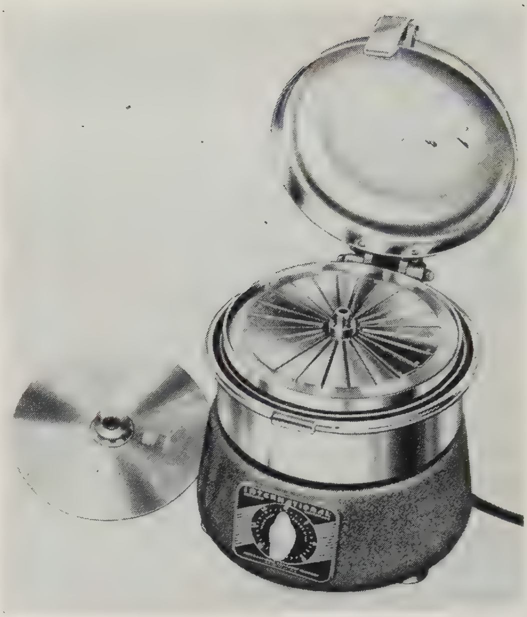

Fig. 362. International microhematocrit centrifuge, showing special hematocrit head, cover, and automatic timing device. (Courtesy International Equipment Co., Boston, Mass.)

$$\text{Vol. packed red blood cells } (\%) = \frac{24}{60} \times 100 = 40\%$$

Microtechnic for hematocrit determination

Equipment:

1. Capillary tubes (heparinized).
2. High-speed centrifuge.
   The Guest-Weichselbaum* centrifuge is excellent. This centrifuge is made with a motor which has a speed of 10,000 r.p.m., and an automatic electric timer built into the apparatus. The special flat head contains notches which permit handling 24 microhematocrit determinations simultaneously. A flush-fitting cover, held in place by a set screw, is furnished with the centrifuge. (Fig. 362.)
3. Plastic hematocrit linear scale for direct reading (Fig. 364).

Procedure:

1. If oxalated venous blood is available, fill the open end of a **plain** capillary tube with blood. If capillary blood is used, fill a **heparinized** capillary tube with the blood sample.
2. Seal the ends of the tube with a lighted match or a burner. Other sealing methods are available, such as rubber cups and special sealing wax.
3. Place the capillary in the centrifuge, the sealed end toward the outside, taking care to balance it against another tube of the same

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*Aloe Scientific, St. Louis, Mo.

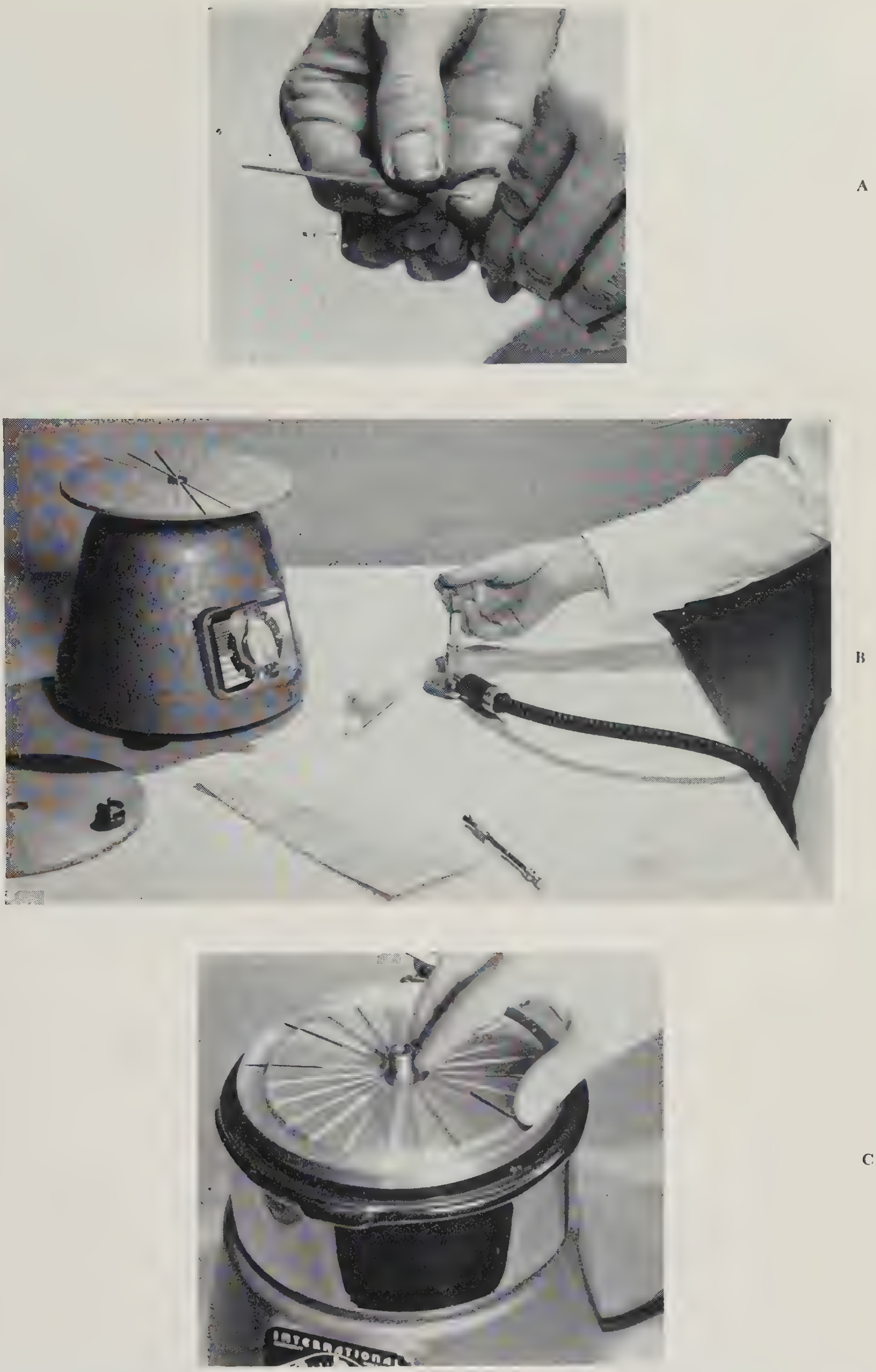

Fig. 363. A, Obtaining finger blood for microhematocrit test. The blood is taken in a capillary tube containing an anticoagulant. **B,** Sealing capillary containing blood preparatory to centrifuging in special hematocrit-head centrifuge. **C,** Placing sealed capillary in special centrifuge head for microhematocrit test. Tubes must be placed opposite each other for balancing centrifuge head. (**A** and **B,** courtesy Aloe Scientific, St. Louis, Mo.; **C,** courtesy International Equipment Co., Boston, Mass.)

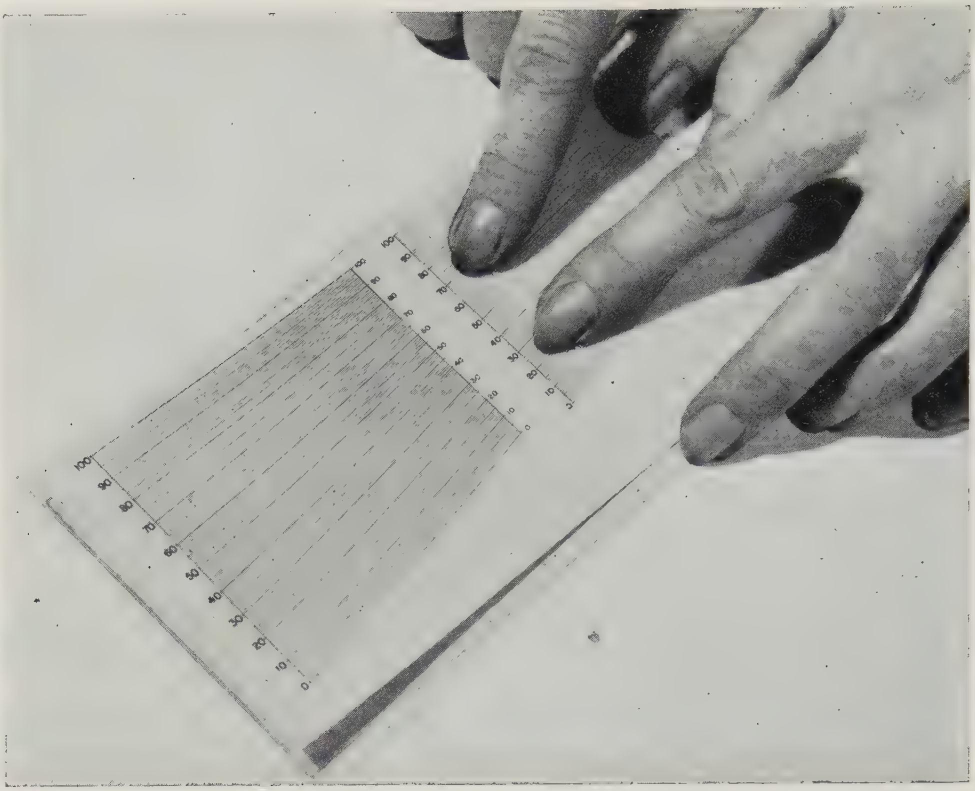

Fig. 364. Special plastic hematocrit linear scale for reading cell vol.% of erythrocytes in microhematocrit test. Capillary tube is placed on scale with bottom of blood column on "0" line and top of column of blood at "100" line. Read the volume of packed cells from scale at left of graphic reader. (Courtesy Aloe Scientific, St. Louis, Mo.)

size on the opposite side of the centrifuge head.

4. Place the cover on the centrifuge and centrifuge, setting the automatic timer at 4 min.
5. Read % blood cell vol. directly from the graphic reader. Hold the tube against the linear chart so that the top of the liquid is exactly at the top line and the bottom of the tube agaisnt the bottom line. Move the tube until it reaches such position and then read vol. packed cells directly from the reader.
6. With a magnifying hand lens, examine the characteristics of the plasma and those of the buffy coat of platelets and leukocytes.

Comment: The advantage of this micromethod is that it requires small quantities of blood and therefore lends itself well to pediatric practice. In emergency situations such as those associated with blood loss and shock, the short centrifuging time offers a distinct advantage over the macrotechnic. Another advantage is that the capillary tubes are inexpensive and disposable.

Interpretation: In anemias generally the hematocrit is reduced below the normal figures, i.e., below 40 vol.% in males and 37% in females. This method is therefore a very simple, readily available method for detecting the presence or absence of anemia. However, the hematocrit alone does not disclose the nature of the anemia and at times may fail to reveal an existing anemia although this is rarely the case.

In polycythemia, the hematocrit value is elevated above 54% in males and 47% in females. The degree of increase, however, does not indicate the nature of the polycythemia, failing to distinguish between polycythemia vera and secondary polycythemia.

In shock associated with surgery, trauma, and burns, hemoconcentration occurs and may be measured by increases in the hematocrit values (rapidly rising).

In conditions associated with hydremia, such as cardiac decompensation, pregnancy, excessive administration of fluids, and other similar states, the hematocrit values may be below normal, and yet no anemia may be present.

ERYTHROCYTE CONSTANTS AND INDICES

After the erythrocyte count, hemoglobin, and hematocrit values in a patient are determined, it is possible to express individual aspects of the red blood corpuscles in either the absolute terms of Wintrobe[107] or the older relative terms. The most commonly used constants are helpful in designating such characteristics of red blood cells as size and hemoglobin content and hemoglobin concentration. In order for these constants to have any real significance, the basic determinations of erythrocyte count, hemoglobin, and hematocrit must be performed accurately. Wintrobe recommends the use of absolute terms for these constants in contrast with the older terms which were relative and were based on percentages:

CONSTANTS RELATING TO ERYTHROCYTE SIZE

Mean corpuscular volume

The mean corpuscular volume (MCV) in cubic microns (cμ) is determined by the following formula:

$$\text{MCV} = \frac{\text{Vol. packed red cells, ml./1000 ml. blood}}{\text{Erythrocyte count millions/c.mm.}}$$

Normal:

Adult males: average 87 cμ; range, 70-94.

Adult females: average 87 cμ; range, 74-98.

Examples:

Normal blood: Hematocrit is 45 vol.%; erythrocyte count is 5,000,000.

$$\text{MCV} = \frac{45 \times 10}{5} = 90 \text{ c}\mu$$

Microcytic anemia: Hematocrit is 22.5; erythrocyte count is 3,500,000.

$$\text{MCV} = \frac{22.5 \times 10}{3.5} = 64 \text{ c}\mu$$

Macrocytic anemia: Hematocrit is 21.2; erythrocyte count is 2,000,000.

$$\text{MCV} = \frac{21.2 \times 10}{2.0} = 106 \text{ c}\mu$$

Volume index

This index corresponds to the mean corpuscular volume. It is determined by the following formula:

$$\text{Vol. index} = \frac{\%\text{ vol. packed erythrocytes}}{\%\text{ erythrocytes}}$$

Normal: Average, 1.0; range, 0.85-1.15.

It is customary to take an arbitrary figure, such as 43.2, for the normal hematocrit and 5,000,000 for the normal red blood cell count to represent 100% in these calculations. As with all such determinations, there is a difference of opinion as to what constitutes 100%. We suggest, for consistency, that the mean average levels given in Table 232 be taken for these determinations.

Table 232. *Mean average normal value of hematocrit and RBC count*

	Hematocrit	*Erythrocytes/ c.mm.*
Adult males	47 vol.%	5,400,000
Adult females	42 vol.%	4,800,000

Examples:

Normal female:

Hct., 40. 40 ÷ 42 is 95% of normal.

RBC, 4,500,000. 45 ÷ 48 is 94% of normal.

$$\text{Vol. index} = \frac{95}{94} = 1.01$$

Normal male:

Hct., 48. 48 ÷ 47 = 102% of normal.

RBC, 5,200,000. 52 ÷ 54 = 96% of normal.

$$\text{Vol. index} = \frac{102}{96} = 1.06$$

Microcytic anemia—male subject:

Hct., 22.5. 22.5 ÷ 47 = 48% of normal.

RBC, 3,500,000. 35 ÷ 54 = 65% of normal.

$$\text{Vol. index} = \frac{48}{65} = 0.74$$

Macrocytic anemia—female subject:

Hct., 21. 21 ÷ 42 = 50% of normal.

RBC, 2,000,000. 20 ÷ 48 = 42% of normal.

$$\text{Vol. index} = \frac{50}{42} = 1.18$$

Comments on determination of size of red blood cells

We recommend determination of the mean corpuscular volume over determination of volume index as an expression of the **mean** size of red blood cells. Certain reservations should be made, however, in accepting mean corpuscular volume figures as necessarily reflecting extreme accuracy. The accuracy of the mean corpuscular volume is only as great as the accuracy of the methods by which the red count and the hematocrit value have been determined.

There are many ways of determining the size of red cells. Perhaps the simplest is to examine a well-prepared blood spread, in which recognition of variations in size as well as the general trend in size is readily perceptible. The blood spread should be used as a check on mean corpuscular volume determinations.

There are other methods for determining the size of red blood cells, but generally speaking, these are of relatively little practical clinical value. Most of these are perhaps of greater historic interest than of practical value. Among the earliest attempts at the measurement of the actual diameter of red blood cells is the **method of Price-Jones,**[108] who made camera lucida drawings of red cells, measuring the actual diameters of the drawings of from 500 to 1000 cells. From these drawings, he constructed curves which are spoken of as the **Price-Jones curves,** showing the distribution of various cell sizes. These curves were so graphed that shifts to the left of the normal distribution curve were seen when the mean diameter of the red cells was diminished, and shifts to the right were seen when the mean diameter was increased.

Three main types of curves are described. The curve of the **normal blood** shows a peak at about 7.2μ. The diameter of red blood cells of **newborn** infants and of young red blood cells **just released from bone marrow** is increased to about 8.2μ. A similar increase in red cell diameter is seen in **macrocytic anemias** which may peak at 9μ. In **severe iron-deficiency anemia** and in **congenital hemolytic jaundice** the peak of the curve is at about 6μ.

As a substitute for the camera lucida drawings there have been devised micrometer disks which can be inserted into the ocular system of a microscope, and direct measurement of erythrocytes can be made with the scale on the micrometer disk. These measurements may be made on a dry stained blood film or on a wet preparation in the counting chamber, there being some shrinkage in the diameter of the red blood cells as seen in the fixed preparation when compared with the wet. In dry preparations, the average red cell diameter is 7.5μ, with a range of 7.2-7.8μ. In wet preparations, the erythrocyte diameter is 8.4μ, with a range of 7.4-9.4μ. Cells smaller than the lower values in the range are considered microcytic, and those larger than the upper figures are macrocytic.

A number of methods have been devised based on the **Pijper principle**[109] which consists essentially of the deflection of white light when passing through a screen to form a spectrum. Halometers have been produced in Europe for a good number of years, and Haden[110] produced an instrument based on this principle which is commercially available. Dingee[111] suggested a method originally described by Pryce.[112] In her ingenious technic, two 25-watt frosted electric light bulbs were placed 28 in. apart on a wall against a black background. A tape measure was fixed between the lights and was held to a stained blood film. When one looks through the blood spreads toward the 2 lights, 2 halos will be seen. The distance between the blood film and the light sources is changed until the red innermost circles of the spectrum of the 2 circles touch. The distance in feet from the lights to the blood film is then determined, and this distance corresponds to the mean diameter in μ of the red blood cells.

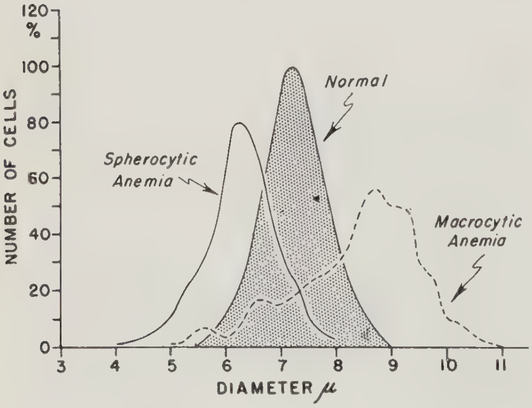

Fig. 365. Price-Jones curves.

A particularly good habit for all technicians is to make an examination of the relative size and distribution of the red blood cell population in making routine blood counts.

CONSTANTS RELATING TO HEMOGLOBIN CONTENT OF ERYTHROCYTES

Mean corpuscular hemoglobin

The mean corpuscular hemoglobin (MCH) indicates the weight of hemoglobin per average erythrocyte. The Wintrobe formula for computing mean corpuscular hemoglobin is as follows:

$$\text{MCH in micro-micrograms } (\mu\mu g) = \frac{\text{Hb in gm./1000 ml. blood}}{\text{RBC in millions/c.mm.}}$$

Normal:
Adult males: 29 μμg, average; range, 25-34
Adult females: 29 μμg, average; range, 24-33
Example:
RBC, 5,000,000; Hb, 15.6 gm./100 ml.

$$\text{MCH } (\mu\mu g) = \frac{15.6 \times 10}{5} = 31.2$$

Color index

This determination corresponds to the mean corpuscular hemoglobin. The formula is as follows:

$$\text{Color index} = \frac{\%\ \text{Hb}}{\%\ \text{erythrocytes}}$$

Normal: Normal color index averages 1.0; range, 0.85-1.15.

In the formula, 5,000,000 erythrocytes/c.mm. is usually considered 100%. As with the determination of volume index and for the sake of consistency, we recommend that the values in Table 233 be used as 100% in calculating the color index.

Examples:
Male subject:
Hb, 14.6 gm.%; 14.6 ÷ 15.8 = 92% of normal.
Erythrocytes, 4,800,000; 48 ÷ 54 = 89% of normal.

$$\text{Color index} = \frac{92}{89} = 1.02$$

Table 233. *Normal values of hemoglobin and number of erythrocytes*

	Hemoglobin	*Erythrocytes/c.mm.*
Adult males	15.8 gm.%	5,400,000
Adult females	13.9 gm.%	4,800,000

Female subject:

Hb, 4.5 gm.%; 4.5 ÷ 13.9 = 32% of normal.

Erythrocytes, 2,100,000; 21 ÷ 48 = 45% of normal.

$$\text{Color index} = \frac{32}{45} = 0.71$$

Comment on mean corpuscular hemoglobin determination

The mean corpuscular hemoglobin generally parallels changes in the mean corpuscular volume. In other words, when the red cells are smaller, the amount of hemoglobin per red cell is less. When the red cells are larger, the amount of hemoglobin is greater. But in both instances, the actual **concentration** of hemoglobin within the red cells may well be the same. In view of this, determination of the mean corpuscular hemoglobin is of relatively little value and adds very little to the knowledge already gained by determination of the mean corpuscular volume. Wintrobe stresses a determination which he feels is much more significant, the mean corpuscular hemoglobin concentration.

Mean corpuscular hemoglobin concentration

Mean corpuscular hemoglobin concentration (MCHC) expresses the concentration of hemoglobin per erythrocyte.

Normal: The normal values for mean corpuscular hemoglobin concentration, expressed in %, is 34% for both males and females, with a range of ±2%.

The formula for determining MCHC is as follows:

$$\text{MCHC } (\%) = \frac{\text{Gm. Hb/100 ml. blood}}{\text{Vol.\% of erythrocytes (Hct.)}} \times 100$$

Examples:

Normal patient:

Hemoglobin, 15.3 gm.%; hematocrit, 45 vol.%

$$\text{MCHC} = \frac{15.3}{45} \times 100 = 34\%$$

Hypochromic anemia:

Hemoglobin, 8.6 gm.%; hematocrit, 36 vol.%

$$\text{MCHC} = \frac{8.6}{36} \times 100 = 24\%$$

Comments on mean corpuscular hemoglobin concentration

Values below 30% of the mean corpuscular hemoglobin concentration are indicative of the **hypochromic** anemias. Values above 36 vol.% are not seen because there is no known condition in which the **concentration of hemoglobin per unit volume** is greater than that which occurs in the normal red blood cell. Therefore, when the term **hyperchromic** is used, it cannot be in the sense that the concentration of hemoglobin in the red blood cell has increased. Wintrobe, as well as others, decries the use of the term hyperchromic. When used in this limited sense of a greater concentration of hemoglobin per unit volume, the term is definitely a misnomer. However, when used to describe red blood cells as seen in the stained blood film, the term hyperchromic is acceptable because these cells do appear to have a greater color than normal red blood cells. This more intense coloring is due to an increased thickness of the red blood cells rather than to an increase in concentration per unit volume of the cells. When the term is understood in this manner, there is perhaps some justification for the continued use of this highly deceptive term.

The hemoglobin concentration of the red blood cell is readily apparent in properly stained blood films. Examination of such films should be made to serve as a check on the determination of mean corpuscular hemoglobin concentration. If one examines enough hematologic material, there will be a fair number of instances in which the blood spread reveals red cells that appear mildly hypochromic, whereas the mean corpuscular hemoglobin concentration is normal, and vice versa. However, when there is a marked change in the concentration of hemoglobin in red blood cells, it will be apparent in both the blood film and the mean corpuscular hemoglobin concentration determination. These two procedures should complement one another in the examination of a blood specimen.

Saturation index

Saturation index corresponds to the mean corpuscular hemoglobin concentration. The formula is as follows:

$$\text{Saturation index} = \frac{\%\ \text{Hb}}{\%\ \text{Hct.}}$$

Normal: Average, 1.0; range, 0.85-1.15.

In this calculation the problem again arises as to which values should be used for the 100%. For the sake of consistency, we recommend the values in Table 234.

Examples:

Normal male:

Hemoglobin, 15.3 gm.%; 15.3 ÷ 15.8 = 97% of normal.

Table 234. *Normal values of hemoglobin and hematocrit*

	Hemoglobin	*Hematocrit*
Adult males	15.8 gm.%	47 vol.%
Adult females	13.9 gm.%	42 vol.%

Hematocrit, 45; 45 ÷ 47 = 96% of normal.

Saturation index $= \frac{97}{96} = 1.01$.

Hypochromic anemia—female subject:

Hemoglobin, 8.6 gm.%; 8.6 ÷ 13.9 = 62% of normal.

Hematocrit, 36; 36 ÷ 42 = 86% of normal.

Saturation index $= \frac{62}{86} = 0.72$.

SUMMARY ON ERYTHROCYTE CONSTANTS

From a clinical standpoint, studies of mean corpuscular volume and mean corpuscular hemoglobin concentration in conjunction with well-prepared stained blood spreads can give inestimable information concerning the anemias. The other constants and indices are interesting but add very little to this. For speed in calculating the absolute constants, Wintrobe[113] presents a nomograph prepared by Robert E. Mason. A similar nomograph is commercially available.*

RETICULOCYTES

Reticulocytes are immature erythrocytes which show a reticulum **when stained by some supravital method. They cannot be demonstrated in a blood spread stained in the routine manner.** Reticulum may be seen in red blood cells both with or without a nucleus. The younger the reticulocyte, the more filamentous and plentiful is the reticulum. In the older reticulocyte, the reticulum may be seen as a single small bluish dot. The reticulum consists of ribonucleic acid, and with the electron microscope it can be seen to form a mitochondrial pattern. For further discussion, see also the section on hemolytic anemias.

The normal reticulocyte count is 0.5 to 1.5%. An increase in reticulocytes is evidence of active regeneration of erythrocytes.

For such a relatively simple procedure, it is amazing how many variations there are for performing this test. We shall present several methods with the admonition that features of any of these procedures may be combined to form another method.

Method of choice: new methylene blue method[114]

Reagent:

1. New methylene blue N solution.
   Dissolve 0.5 gm. new methylene blue N, 1.4 gm. potassium oxalate, and 0.8 gm. sodium chloride in 100 ml. distilled water. Filter just before use.

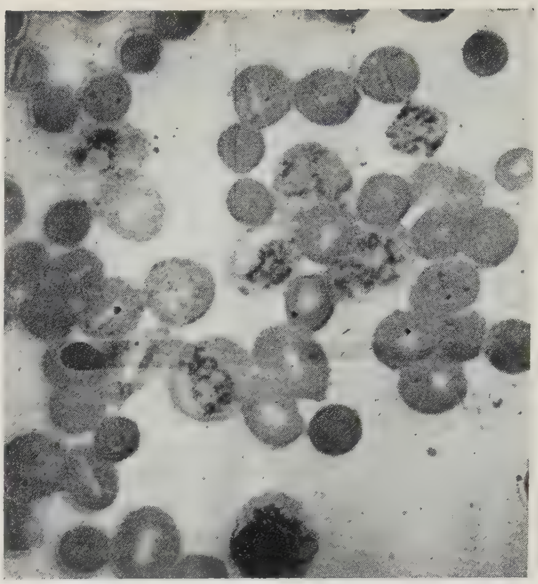

Fig. 366. Hemolytic icterus. Blood film, supravital stain, showing special staining of reticulocytes. (×950.)

Blood specimen: Venous oxalated blood can be used if used within 2-3 hr. after withdrawal from patient. Peripheral blood can also be used.

Procedure:

1. If venous blood is used, add 3 or 4 drops methylene blue N solution to an equal quantity of well-mixed blood in a small test tube and mix.
2. If peripheral blood is used, draw the methylene blue N solution into a white counting pipet until the bulb is half full, admit a small air bubble into the stem, and then draw up the blood from a fresh puncture until the bulb is filled. Mix.
3. Allow to stand 10 min.
4. Place 1 drop dye-blood mixture on the end of a clean glass slide and spread in a manner similar to that for preparing a blood film. Make several slides.
5. Dry rapidly by waving slides in the air vigorously.
6. The slide may be examined under oil immersion without further preparation, or it may be counterstained with Wright stain or Wright-Giemsa combination.

Counting reticulocytes: Using the 4-field meander method of counting, count 1000 red blood cells (250 per field), simultaneously enumerating those erythrocytes containing a reticulum (reticulocytes).

Calculation:

$$\text{Reticulocyte count in } \% = \frac{\text{No. reticulocytes counted}}{\text{No. RBC counted}} \times 100$$

Example:

46 reticulocytes counted; 1000 erythrocytes counted.

$$\% \text{ reticulocytes} = \frac{46}{1000} \times 100 = 4.6\%$$

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*Best anemia classifier, New York, 1952, Clay Adams Co.

Method of Cook, Meyer, and Tureen

Reagents:

1. Solution A—1% solution of brilliant cresyl blue in saline.
   Dissolve 1 gm. brilliant cresyl blue by grinding in a mortar using 100 ml. warmed 0.85% sodium chloride. When cool, filter into a glass-stoppered bottle.
2. Solution B—1% solution of potassium oxalate in saline.
   Dissolve 1 gm. of potassium oxalate, neutral, in 100 ml. 0.85% sodium chloride. Filter into a glass-stoppered bottle.

Procedure:

1. Place 5 drops solution A in a watch glass.
2. Add 1 drop solution B and mix.
3. Make the puncture of the skin in the usual manner.
4. Using a Sahli pipet or capillary tube, draw up some blood and an equal amount of the mixed stain. **To avoid precipitates, do not use an excess of the stain.**
5. Mix thoroughly by alternately blowing out onto a glass slide and drawing up into the pipet. This should be done several times to ensure a good stain.
6. Allow to stand 5 or 6 min. covered with a Petri dish to prevent evaporation.
7. Place a small drop mixture of blood and stain upon the end of a glass slide and spread with a cover slip in the manner of making ordinary blood films.
8. Dry quickly to prevent distortion of the cells.
9. This slide may be examined immediately or counterstained with either Wright or Giemsa stain. If the Giemsa stain is used, the slides are fixed for 3 min. in methyl alcohol preparatory to staining.
10. Proceed as in new methylene blue method.

Comment: Although Cook, Meyer, and Tureen used the classical brilliant cresyl blue, the newer dye, new methylene blue N, could be substituted for it.

Rapid method of Schilling

This method is not permanent.

Reagent:

1. Alcoholic solution of brilliant cresyl blue, 1%.
   Dissolve 1 gm. brilliant cresyl blue in 100 ml. absolute alcohol, grinding the dye in a mortar. Filter after 24 hr. Keep in a tightly stoppered bottle with a glass rod attached.

Procedure:

1. Dip the glass rod into the solution. Spread a thin layer over a fat-free slide and allow to dry in the air.
2. Puncture the patient's skin in the usual manner and place a very small drop of blood upon the layer of dye.
3. Place a large cover glass over the drop of blood. The drop of blood must be so small that it does not spread to the edges of the cover glass.
4. Examine with oil immersion objective after about 10 min.
5. Proceed as in new methylene blue method.

Sabin method[115-118]

Materials:

1. Stock solutions.
   (a) Prepare neutral red special for supravital staining by dissolving 125 mg. neutral red in 50 ml. neutral absolute ethyl alcohol.
   (b) Prepare Janus green special for supravital staining by dissolving 125 mg. dye in 62.5 ml. neutral absolute alcohol.
2. Working solution.
   (a) Dilute 1.1 ml. stock solution neutral red in 10 ml. neutral absolute ethyl alcohol.
   (b) Add 8 drops stock solution Janus green.

Preparation of slides:

1. Flood grease-free clean slides with the working solution of Janus green-neutral red solution.
2. Drain off excess dye into working solution bottle.
3. Wipe off every drop of solution on dependent portion of slide.
4. Dry quickly by rapidly waving slide in the air or by holding it over a gentle flame.
5. Mark stained side of slide.

Slides prepared in this manner may be stored for future use, provided that this storage is free from dust. Stained films must be thin and must show even distribution of dye. Discard slides that are too thickly stained or that show uneven distribution of the stain.

Procedure:

1. Place a small drop blood on a clean cover slip and invert upon the stained surface of a neutral red-Janus green slide.
2. Seal edges of cover slip with petrolatum or a mixture of petrolatum and paraffin.
3. Allow preparation to stand 10 min. before examining for reticulocytes. The slide may be examined up to 24 hr. later if kept refrigerated at 4° C.
4. Examine under oil immersion.
5. Count reticulocytes as outlined under new methylene blue method.

Examination of Sabin-stain slides for supravital staining of leukocytes: Neutrophilic leukocytes (juvenile, stab cells, and segmented forms) move constantly with characteristic ameboid motion. The granules are numerous, small, and pale red and are constantly streaming through the cytoplasm. The nucleus usually is in the rear of the moving cell. There are present also one or more rounded bodies, presumably digestive vacuoles, which take the stain slowly. They indicate phagocytic activity, according to Sabin. They vary in color. Some leukocytes are round and motionless, the structureless nucleus nearly filling the cell and the granules, although distinctly visible, not taking the stain. These are dead or dying neutrophils. During the middle of the day, the number of these dead neutrophils is greatest. They are increased by faulty technic.

Eosinophils and basophils show red granules. The granules of the basophils are smaller than those of the eosinophils, are uneven in size, and have different staining characteristics. There are no digestive vacuoles in either cell. Eosinophils are actively motile; basophils are sluggish.

Monocytes with oval nuclei are rounded and practically nonmotile. The very fine salmon-colored granules are grouped around a clear spot, the centrosphere. There are a variable number of larger red bodies, presumably digestive vacuoles. Those monocytes with lobulated or curved nuclei are irregular in shape, usually elongated, and are sluggishly motile. The red vacuoles usually displace or obscure the fine granules.

The **lymphocytes** show a clear cytoplasm with a few small vacuoles which take the red stain. When Janus green is added, a clump of blue mitochondria may be seen opposite the nucleus. The nucleus is oval or indented. There is no motility although a few moderate-sized lymphocytes show sluggish motility.

Comment: Preparations of this kind are commonly referred to as supravital. These may be used for differential counting and blood platelet estimations, as well as for reticulocyte counting. Some workers prefer this method to the fixed spread technic. One major objection is the lack of permanence of the preparations so that preparations must be examined within 24 hr., and there are no slides available for future reference.*

The supravital method is also useful in the identification of monocytes and monoblasts and in the diagnosis of acute monocytic leukemia. It also aids in distinguishing micromyeloblasts from lymphocytes. Especially attractive is the combination of supravital staining and phase contrast microscopy as it has been described by Ackerman and Bellias. (See comments on phase microscopy.)

Comments on reticulocytes

An increase in the reticulocyte count is evidence of active regeneration of erythrocytes. The most classic reticulocytosis is that which occurs after the administration of erythrocyte maturation factor (liver extract or vitamin B_{12}) to patients with pernicious anemia in relapse. This reticulocytosis usually reaches a level of 20% by the seventh to tenth day after treatment is initiated and then drops. In part, the height of response is governed by the degree of the anemia.

Patients with iron-deficiency anemia, such as occurs with blood loss, show reticulocytosis after iron therapy. The degree of reticulocyte response is usually less than that seen in pernicious anemia under treatment. Diseases associated with hemolytic crises, such as congenital hemolytic icterus, sickle cell anemia, and erythroblastosis fetalis, may show exceedingly high (40-60%) reticulocyte counts.

Increase in reticulocytes:
Physiologic:
- At birth
- Menstruation
- Pregnancy

In disease:
- Erythropoietic response following treatment of anemias with Fe, vitamin B_{12}, folic acid, etc.
- Blood intoxication
- Erythroblastic anemias
- Granulomatoses
- Hemolytic anemias, very high
- Following hemorrhage
- von Jaksch's disease
- Kala-azar
- Lead poisoning, very high
- Leukemia
- Malaria
- Pernicious anemia and sprue following folic acid therapy (as a response to treatment) and following vitamin B_{12} therapy
- Polycythemia vera
- Relapsing fever
- Sickle cell anemia
- Splenic tumor
- Frequently in trypanosomiasis
- In typhoid fever and brucellosis occasionally
- Many parasitic infestations

Normal or increased:
- Normal individuals

Low or absent:
- Idiopathic aplastic anemia
- Acute benzol poisoning
- In anaplastic crises of hemolytic anemias

Value of reticulocyte counts:

1. To determine the response of the pernicious anemia patient to vitamin B_{12} therapy or to confirm a diagnosis of pernicious anemia by therapeutic response
2. To aid in the diagnosis of lead poisoning and hemolytic icterus.
3. To determine whether regeneration of erythrocytes is proceeding normally and whether it occurs at all, which is of value in establishing a diagnosis of aplastic anemia.
4. To aid in the prognosis of acute hemorrhage, in which an increase denotes regeneration of erythrocytes.

For further comments on reticulocytes, see discussion of reticulocytes (p. 1132) and the discussion of stained cells (p. 1133).

FRAGILITY OF ERYTHROCYTES[120]

OSMOTIC (SALINE) FRAGILITY

General considerations

The red blood cell is surrounded by a semipermeable membrane, permeable to water and electrolytes. If a red blood cell is placed in a hypotonic solution, osmotic equilibrium will be established by drawing water into the cell, which thus swells, the degree of swelling depending on the osmotic pressure gradient. In a hypertonic solution the cell will lose water and shrink. In general, the osmotic pressure for substances in solution follows Boyle's law of gases, but in the case of red blood cells several modifying factors play a role, such as the degree of crystallization of hemoglobin, the degree of oxygen saturation, the rate of penetration, the activity of transfer enzymes, the pH and temperature of the solution, and the total red blood cell count. Because of these many variables, osmotic fragility measurements should be strictly standardized and a normal control run with each test.

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*Permanent slides may be prepared using the method of Cook, Meyer, and Tureen or the new methylene blue method.

The red blood cell surface consists of lipid and protein interrupted by pores through which most of the hemoglobin escapes when the cell membrane is stretched in hypotonic solutions. Not all the hemoglobin escapes; some of it remains within the cell attached to the stromal network.

As will be shown in the discussion of hemolytic anemias, the hemolytic phenomenon is closely related to the red cell structure. The penetrating water and the resulting electrolyte displacement produce changes in the hemoglobin molecule so that it can escape through the pores of the cell membrane.

Normal erythrocytes suspended in 0.85% sodium chloride solution remain unchanged for hours. As the saline concentration is reduced, initial hemolysis will be noted at 0.44-0.46% and complete hemolysis at 0.32-0.30%. In vitro, this process is reversible to a certain degree and can be followed by observing the normal red blood cell becoming crenated, then discoid, and finally spherical when exposed to hypotonic solutions. When the hemoglobin leaves the cell, the stroma, the ghost, remains. As spherocytosis represents one of the stages of hemolysis, naturally occurring spherocytes exhibit an increased osmotic fragility, hemolysis often beginning at 0.70% concentration of sodium chloride and becoming complete at 0.40% concentration. Their resistance to hemolysis is thus markedly lowered.

Whenever spherocytes occur, such as in congenital spherocytosis, acquired hemolytic anemia, hemolytic disease of the newborn due to A-B-O incompatibility, the osmotic fragility is increased. Microspherocytes have been described in chemical poisoning such as naphthalene and in burns. Decreased osmotic fragility, i.e., increased resistance, is seen in thalassemia, sickle cell anemia, erythremia, and jaundice. The initial hemolysis may be at 0.50% concentration of saline solution, and cells may still be intact at the bottom of the last tube containing 0.07% saline solution. The predominant cell in these conditions is thin and flat (target cell).

Presumptive test for increased osmotic fragility

1. Tube 1: 1 ml. 0.85% saline solution.
2. Tube 2: 1 ml. 0.50% saline solution.
3. To each tube add 0.1 ml. oxalated venous blood.
4. Mix and centrifuge.

Increased osmotic fragility is indicated by hemolysis in tube 2.

Wintrobe modification for osmotic fragility[120]

Materials:

1. Stock solution 0.75% saline.
   Prepare with precision 0.75 gm. NaCl/100 ml. distilled water.
2. Fresh venous 3:2 oxalated blood.
   A 0.5 ml. specimen from patient and from normal control.
3. Series of 18 test tubes in a rack numbered from 48 down to 14 in steps of 2, e.g., 48, 46, 44, etc., to 14.
4. Arrange a duplicate series of tubes for a control.
5. Serologic pipet, 5 ml., graduated in 0.1 ml.
6. Distilled water.

Preparation of different concentrations of saline:

1. Into each tube, add 0.75% saline solution in amounts equal to $\frac{1}{10}$ of the number on the tube; e.g., in tube 48, place 4.8 ml. of saline, in tube 46, 4.6 ml., etc.
2. To each tube add sufficient distilled water to bring the total to 5 ml.
3. The concentration of sodium chloride in each tube will be $\frac{1}{50}X \times 0.75\%$, where X represents the number of ml. of saline in the tube. In simpler terms, multiply the number on the tube by 0.015 to obtain the concentration of sodium chloride in the tube.

Example:

Tube 48 = 48 × 0.015 = 0.72% saline.

Mix all tubes thoroughly.

Procedure:

1. To each tube add 0.2 ml. patient's blood.
2. Prepare a similar series using normal blood as a control.
3. Shake tubes gently to ensure thorough mixing.
4. Place in a refrigerator for 2 hr.
5. Examine the supernatant fluid of the tubes for beginning hemolysis (faintest pink) and for complete hemolysis (no sediment of erythrocytes remaining in the bottom of the tube). Gentle centrifugation (1000 r.p.m. for 5 min.) aids observation of the supernatant fluid.

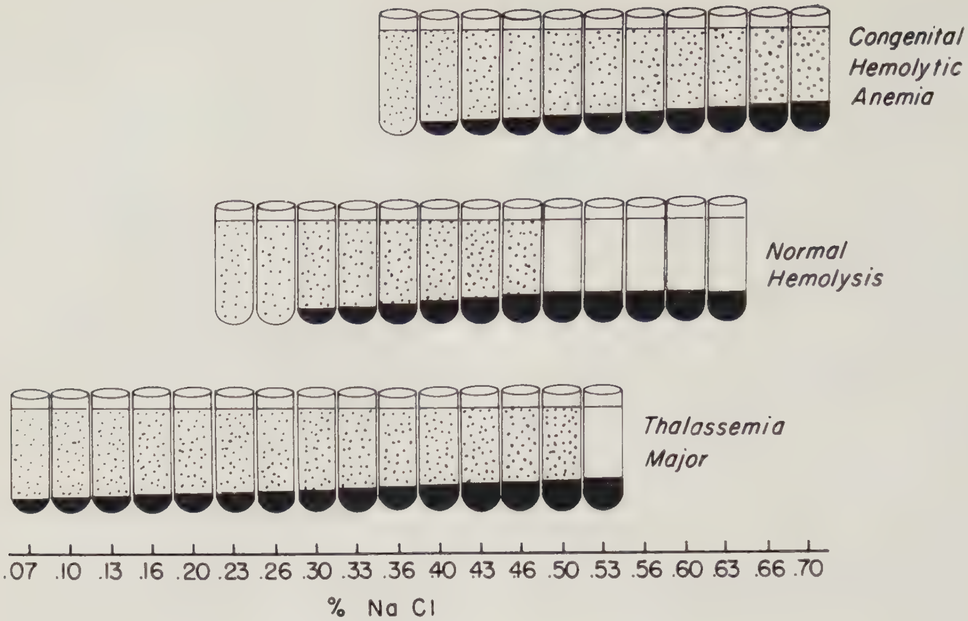

Fig. 367. Osmotic fragility (Wintrobe).

Incubation at 37° C. increases the sensitivity of this method. If incubation is used, the method is modified as follows:

Modification:

1. Defibrinate 10 ml. sterile venous blood in an Erlenmeyer flask containing 15 glass beads (3 mm. in diameter each) or use 5 ml. heparinized or oxalated (hematologic oxalate) blood.
2. Incubate at 37° C. for 24 hr. and then determine fragility as just outlined.

Normal: Hemolysis begins at 0.45-0.39% saline, is well marked at 0.42-0.36%, and is complete at 0.33-0.3%.

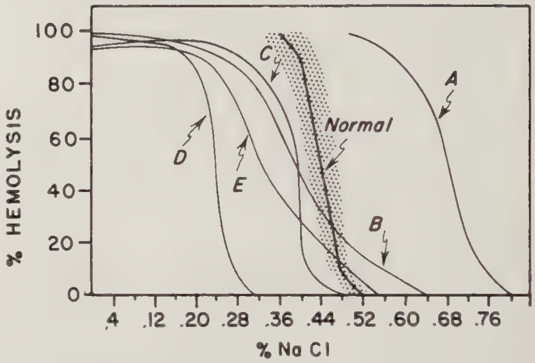

Fig. 368. Erythrocyte osmotic fragility curves (Dacie method).

Table 235. *Saline dilutions*

NaCl concentration (%)	*Vol. stock solution*	*NaCl concentration (%)*	*Vol. stock solution*
0.15	3.0	0.50	10.0
0.20	4.0	0.54	10.8
0.26	5.2	0.58	11.6
0.30	6.0	0.62	12.4
0.34	6.8	0.66	13.2
0.38	7.6	0.75	15.0
0.42	8.4	0.85	17.0
0.46	9.2		

Method of Dacie[121]

Materials:

1. Acid washed test tubes (30).
2. 5 ml. volumetric pipets.
3. Sahli hemoglobin pipets.

Principle: Blood is diluted in hypotonic saline solution and is buffered to pH 7.4 with phosphates. A series of tubes is prepared, each containing a different concentration of saline solution. After standing at room temperature the unlysed cells and ghosts are removed by centrifugation, and the degree of hemolysis in each tube is determined spectrophotometrically. A tube in which blood is diluted in water so that all of the cells lyse is used as a 100% hemolysis standard.

Reagents:

1. Stock solution.
   Dissolve 45 gm. NaCl, 6.83 gm. Na_2HPO_4, and 1.08 gm. $NaH_2PO_4 \cdot H_2O$ in distilled water and dilute to 1 L. This stock solution is osmotically equivalent to 5% NaCl and is diluted to prepare working solutions.
2. Working solutions.
   Dilute the volumes of the stock solution to a total volume of 100 ml. with distilled

water, as in Table 235. These standards are stable indefinitely if refrigerated.

Procedure:

1. Use heparinized blood.
2. Measure 5 ml. each NaCl solution into a properly labeled tube and at the same time measure 5 ml. distilled water into a tube and label 100% hemolysis.
3. Allow all tubes to warm to room temperature.
4. Add 0.02 ml. blood to each tube, mix the contents gently, and allow to stand at room temperature for 60 min.
5. Centrifuge 5-10 min. at 2000 r.p.m. and transfer the supernatant solutions to cuvets.
6. Set the wavelength dial of the spectrophotometer at 540 mμ, or if a filter photometer is used, insert a 520 or 540 mμ filter.
7. Set the instrument at zero optical density using the supernatant fluid from the 0.85% NaCl tube as reference and determine the optical density of each supernatant fluid.

If the scale of the photometer used is graduated only in % T, these values should be obtained and then converted to OD.

Determine the % lysis in each NaCl solution by dividing its OD by that of the tube in which 100% hemolysis occurred and multiplying by 100.

Example:

Reading of 0.46% NaCl = 0.240
Reading of 100% lysis tube = 0.600

If % hemolysis in each tube is plotted against its NaCl concentration, a sigmoid-shaped curve will be obtained.

Discussion: There are 3 primary influences that affect the accuracy of this determination: (1) the relative volumes of blood and saline, (2) the final pH of the blood-saline suspension, and (3) the temperature at which the tests are carried out.

The proportion of blood to saline chosen here is so small that the added blood has no appreciable effect on the osmolarity of the saline solutions; yet it is high enough to permit accurate photometric readings. However since so little blood is added, it is necessary to buffer the saline solutions used.

The effect of alterations in pH on osmotic fragility is perhaps more significant than any other single variable; a shift of 0.1 of a pH unit is equivalent to altering the tonicity of the saline solutions by 0.01%, the fragility of the erythrocytes being increased as the pH falls. A rise in temperature decreases the fragility, a rise of 5° C. being equivalent to an alteration in tonicity of about 0.01%. If very precise results are desired, the tubes may then be incubated in a water bath, preferably at 30° C.

The method of expressing the results of this test has varied from laboratory to laboratory. Plotting the data as suggested permits the ready determination of the distribution of the relative susceptibility to lysis of the entire erythrocyte population. It is suggested that the two most meaningful parameters are the concentration of saline in which 10% of the cells are hemolyzed and the concentration in which 90% are hemolyzed.

Normal:

10% hemolysis = 0.48-0.44% saline
90% hemolysis = 0.4-0.36% saline

Conclusions: Dacie's method[121, 122] is probably the best of a long line of possible procedures. An analysis of the curve shows it to be sigmoid in type. The slowly rising lower limb corresponds to the beginning hemolysis and swelling of the red blood cells. The steep middle portion represents the main escape of the hemoglobin, whereas the upper limb represents complete hemolysis.

In vivo the red blood cells are surrounded by an isotonic medium and therefore are not subjected to the pressure differences of the osmotic fragility tests. The mechanical fragility test is supposed to recreate the mechanical stresses to which the red blood cells are subjected in circulation and is therefore considered to be more physiologic.

MECHANICAL FRAGILITY [123-125]

Materials:

1. Mechanical shaking device.[126]
2. 50 ml. Erlenmeyer flasks.
3. Glass beads 4 mm. in diameter.
4. Incubator.
5. Sterile applicators.
6. Centrifuge and hematocrit tubes.
7. Distilled water.
8. Sodium chloride solution, 1.25%.
   Accurately weigh 1.25 gm. sodium chloride on an analytical balance and dissolve in distilled water in a 100 ml. volumetric flask. Dilute to 100 ml. with distilled water.

Procedure:

1. Draw 5 ml. blood with sterile precautions.
2. Transfer to a sterile 50 ml. Erlenmeyer flask containing glass beads and defibrinate by gentle rotation.
3. Divide the defibrinated blood into 2 parts. Use 1 part for immediate determination of mechanical fragility and incubate the other portion at 37° C. for 24 hr., and then determine the mechanical fragility of this specimen.
4. Adjust the hematocrit of the defibrinated blood specimen to 35 vol.% by adding or removing plasma.

Preparation of rotated sample (S):

1. Place 0.5 ml. adjusted sample in a 50 ml. Erlenmeyer flask in the shaking device. The flask should contain 8 glass beads of 4 mm. diameter.
2. Rotate in a mechanical rotor at 100 r.p.m. for 60 min.

3. Take duplicate 0.1 ml. samples of this rotated specimen and add each one to 1 ml. 1.25% sodium chloride solution in a centrifuge tube.

Preparation of sample showing complete hemolysis (H):

1. Add 0.1 ml. adjusted blood specimen to 1 ml. distilled water in a centrifuge tube.

Preparation of sample showing no hemolysis (O):

1. Add 0.1 ml. adjusted blood sample to 1 ml. 1.25% sodium chloride solution in a centrifuge tube.
2. Centrifuge samples S, H, and O at 1000 r.p.m. for 5 min.
3. Remove 0.5 ml. supernatant fluid of each centrifuged specimen and add this to 9.5 ml. distilled water.
4. Determine the hemoglobin of each diluted specimen in a photoelectric colorimeter.

Calculations:

$$\text{Percentage of mechanical fragility} = \frac{S - O}{H - O} \times 100$$

S = average of duplicated rotated specimens.

The sensitivity of the test can be increased by incubating the blood for 12 hr. at 37° C.

The mechanical fragility test may attain some importance in cardiac surgery, as apparently heparinized normal blood which is used in these procedures shows marked variations in mechanical fragility.

Table 236. *Normal range of mechanical fragility*

	Unincubated		*Incubated*	
Hemolysis	*Mean* %	*Range* %	*Mean* %	*Range* %
Newborn infants	7.1	1.1 to 11.5	13.4	3.7 to 21.6
Children	4.0	0 to 6.0	10.5	2.5 to 23.0
Adults	4.0	1.3 to 6.7	10.8	4.6 to 19.0

ROUTINE STAINING OF BLOOD FILMS

Preparation of glassware

Slides and cover slips: The cover glasses recommended are ⅞ in. wide, size B, square.

All new slides and cover glasses must be cleaned thoroughly before use to render them fat-free. Make a weak solution of a detergent (Alconox, etc.) by using about half a teaspoonful of detergent to a pint of warm water. Admit the slides and cover glasses into this solution, one at a time, so that the entire surface of each is in contact with the wetting agent. Allow them to remain in the solution for a period of 15 min. to a few hours. Do not leave them in too long because they become coated and cannot then be used.

Wash very thoroughly preferably in running water **until all traces of the detergent have been removed.** Place them in warm water and dry immediately, using a soft cloth. Linen which has been washed many times makes an excellent towel for drying slides. Use enough pressure in drying the slides to remove any traces of fat which may remain on the slides. Have clean slides and cover glasses on hand at all times in the laboratory. Satisfactory precleaned slides can be purchased.

If slides have been used for oil immersion magnification and are to be kept, dip them in xylol to remove the oil, then hold them at an angle and blow down the slide, blowing the xylol to the bottom of the slides, and dry the drop of xylol that remains. These slides can then be stored or filed for future reference.

If used slides are to be washed and reused, remove the oil with a cloth containing a few drops of xylol and clean in the detergent as just directed. It is recommended that slides should be used only once.

With this method no boiling, washing with soap, or using of ether and alcohol is necessary. Slides are clean and fat-free without further processing. Do not, however, allow the detergent to dry on the slides.

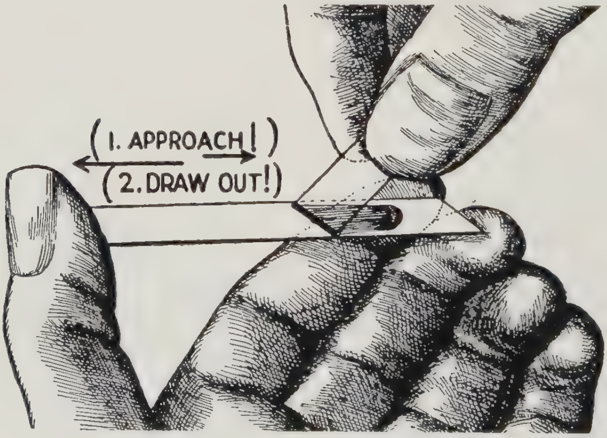
(1. APPROACH!)
(2. DRAW OUT!)

Fig. 369. Correct method of making the film. (From Schilling, V.: The blood picture, St. Louis, The C. V. Mosby Co.)

BLOOD STAINS

Giemsa stain

Considerable difficulty has been encountered by many workers in the manufacture of a serviceable Giemsa stain. The formula of R. D. Lillie has points of superiority over the original Giemsa stain and is available commercially.*

Giemsa stain—formula of R. D. Lillie[127]

Azure B eosinate:

1. Dissolve 10 gm. methylene blue, 85-88% dye content, in 600 ml. distilled water in a mortar. Add 6.8 ml. concentrated sulfuric acid, sp. gr. 1.835-1.84.
2. Bring to a boil.
3. Add 2.5 gm. potassium dichromate dissolved in 25 ml. distilled water.
4. Boil 20 min.
5. Cool to 10° C. or lower. Place in a refrigerator overnight.
6. Add 17.5 gm. sodium bicarbonate slowly with frequent shaking.
7. Add a 5% solution eosin Y of about 90% dye content and shake constantly until the margin of the fluid appears pale blue or bluish pink and an eosin halo is observed on filter paper. About 205 ml. will be required. Three fourths of this amount can be added at once.
8. Filter at once, preferably on vacuum funnel with hard paper.
9. When fluid has been drawn through and the surface begins to crack, add 100 ml. distilled water, let drain, and wash again with a second 100 ml. distilled water.
10. Open out the filter paper and let it lie on a larger piece of filter paper or a paper towel and dry overnight on a warm plate or in an incubator at 37° C. The drying may be accelerated by using two 100 ml. portions of acetone or preferably 95% alcohol as washes after the second wash with water. Drying at 55°-60° C. has been tried and produces quite a little alteration of the thiazin dye; less alteration occurs if acetone or alcohol washes are used, and the heating is limited to 2-3 hr. The resultant dye is the **crude azure B eosinate.**

Azure A eosinate:

1. Proceed as for the azure B eosinate, but take 5 gm. potassium dichromate in place of 2.5 gm. and dissolve it in 50 ml. distilled water.

Methylene blue eosinate:

1. Dissolve 10 gm. methylene blue in 600 ml. cold distilled water.
2. Precipitate as before with 5% eosin, filtering and drying as just described.

Methylene blue:

1. Use certified methylene blue chloride.

Solvent:

1. Equal parts of glycerin and methyl alcohol, certified.

Lillie stain:

1. Grind the azure B eosinate, azure A eosinate, methylene blue eosinate, and methylene blue (when necessary) separately into fine powder, using separate clean mortars.
2. Weigh 840 mg. azure B eosinate, 140 mg. azure A eosinate, 420 mg. methylene blue eosinate, and 280 mg. finely ground methylene blue.
3. Mix the 4 powders thoroughly and pour over the surface of 200 ml. solvent in a brown bottle or flask. Cork the container.
4. Allow the powder to settle into the solvent gradually.
5. Shake frequently for 2 or 3 days, keeping the bottle between 50° and 60° C. between shakings.

If the bottle is tightly stoppered, there will be little or no loss from evaporation, and if there is, the fluid level can be restored by addition of methyl alcohol provided the fluid level was marked on the outside with a wax pencil or a piece of adhesive.

The glycerin should be neutral, anhydrous, and of the purest grade obtainable. Use methyl alcohol special for blood work.

Filter the stain through Whatman No. 5 filter paper into clean, dry, dark bottles. The stain is ready for use and is stable. Use as for Giemsa stain, original formula.

Wright stain

Wright dye:

Aqueous solution of sodium bicarbonate, 0.5% . . .	100 ml.
Methylene blue	1 gm.

1. Grind in a mortar until all the methylene blue is in solution.
2. Place in Erlenmeyer flask to a depth of not more than 6 cm.
3. Heat in an Arnold steam sterilizer at 100° C. for 1 hr.
4. Cool. Filter through filter paper. Save the filtrate.
5. To every 100 ml. filtrate add 500 ml. 0.1% aqueous solution yellowish water-soluble eosin (eosin Y). Add the eosin solution slowly, stirring constantly, so as to form a precipitate. Test for a free eosin ring by placing a drop of the mixture on a piece of white filter paper and drying. The ring appears as a faint pink halo around a lavender spot. If the ring does not appear, add more 0.1% aqueous eosin until the ring forms. The center spot should be purple, not blue.
6. Filter through Whatman No. 5 filter paper, discarding the filtrate. Fold paper into 4 parts.
7. Save the precipitate on the filter paper.
8. Dry the precipitate in an incubator. This may require several days.

This is the stock stain. Place in a dark bottle labeled Wright dye. This dye is stable.

Wright stain solution:

1. All glassware must be absolutely dry.
2. Dissolve 0.1 gm. Wright dye, above, in 60 ml. methyl alcohol, acetone-free. The dye must be ground in a mortar, and the alcohol added slowly until all the dye is in solution. As much as 0.3 gm. may be used if desired.

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*Gradwohl Laboratory Supply Co., St. Louis, Mo.

3. Place in a dark bottle.
4. Filter through filter paper after 24 hr.

The solution is not always stable.

Test and label the bottle with the timing as determined by the test.

Both stains are best purchased ready-made from a reliable supplier.

PREPARATION AND STAINING OF BLOOD FILMS FOR ROUTINE DIFFERENTIAL COUNTS

Prepare the patient (p. 1101), producing hyperemia. Make the puncture and discard the first 1 or 2 drops of blood.

Hold the slide between the thumb and middle finger of the left hand and pick up a very small drop of blood with the slide so that it is about ¼ inch from the end. To avoid endothelial cells do not allow the slide to come in contact with the skin.

Hold the cover glass between the thumb and the first finger of the right hand. Approach the drop with the cover glass at an angle of 30°. When the cover glass touches the drop of blood, allow the blood to run across the edge of the cover glass and then pull it across the slide to form a thin layer. Do not put any tension on the cover glass!

The film should not touch the edges of the slides; i.e., it should be margin-free. Scratch the patient's name in the center or the extreme end of the film as a permanent record. Let the films dry. In summer, cover the spreads to protect from flies, etc. Several spreads should be made at the same time.

The margin-free film is recommended because, when the blood film is allowed to spread to the edge of the glass slide, the large cells tend to accumulate in groups at the edges, and the small cells, such as small lymphocytes and normoblasts, go to the center. With a combination of margin-free films and the 4-field meander method of counting the cells, all spreads taken from the same individual at the same time by different individuals who know how to identify the cells should show the same differential count. The personal element, in other words, has been reduced to a minimum.

If the blood does not flow freely, remove the dried blood from the skin, gently wash the ear with a small amount of ether, and allow to dry. If the blood does not flow freely now, a new puncture must be made. Never squeeze the ear to produce a flow of blood.

Do not use too large a drop of blood, or the spread will be too thick. Do not pull the drop too rapidly, as the film will tend to become thick. If the film is made too rapidly, it will not extend to the end of the glass slide.

STAINING BLOOD FILMS

Giemsa stain*

Steps in technic using Giemsa stain

1. Fix blood film in methyl alcohol 3-5 min.
2. Allow to dry.
3. Neutralize the distilled water.
4. Dilute the Giemsa stain, 1 drop for each ml. neutral water.
5. Cover slides with diluted stain for 30 min.
6. Tip up slides and wash with neutral distilled water.
7. Allow to dry in the air.

The stock Giemsa stain keeps indefinitely. The diluted Giemsa stain must be prepared just before staining. The container in which the stain is diluted must be free of acids and alkalies.

It is well always to dilute the stain in the same container and after using to rinse the container thoroughly in tap water, then in neutral distilled water. The small amount of dye which adheres to the glass is not objectionable; in fact, the stain is better if made in a cylinder or flask which has been used for some time for Giemsa stain. The container should be cleaned from time to time to remove precipitates, after which it is neutralized.

Neutralizing water in staining of blood smears

Neutral distilled water is to be used in the routine staining process.

As Simmons[128] states: "A pH of 6.8 is usually satisfactory. Thoroughly mix the two salts, as shown in Table 237, in a mortar and use 1 gm. to 2000 ml. distilled water. It may be necessary to vary the amounts of phosphates to obtain the desired results. Red is increased by lowering and blue is decreased by raising the pH.

Table 237. *Buffered water (for use with Wright stain)*

pH	$Na_2HPO_4 + 2H_2O$ (gm.)	KH_2PO_4 (gm.)
6.2	1.816	9.504
6.5	2.723	8.316
6.8	4.539	5.940
7.0	5.447	4.752
7.2	6.355	3.564
7.4	7.262	2.376

"Microscopically, a properly stained blood smear will show red cells with a copper or tan color; lymphocytes with a pale blue cytoplasm and a dark blue nucleus; and neutrophiles with a finely granular pink cytoplasm and a dark purplish-blue nucleus. If these characteristics are not distinct, prepare additional slides."*

A simpler and better way of neutralizing water employs hematoxylin as an indicator. Water neutral to hematoxylin in our hands has proved better suited to staining blood cells than has water with a pH of 6.4. We have found that water which is neutral with the use of hematoxylin as an indicator has a pH of 6.8-7.0. This, therefore, gives a better approximate neutrality point than the buffer methods alluded to. The settlement of this neutrality point is especially necesssary in dealing with spreads for the diagnosis of malaria, particularly thick spreads and thick drops. Buffer tablets to be added to the water are commercially available.

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*Certified Giemsa stain is made by the Gradwohl Laboratory Supply Co., St. Louis, Mo.

*From Simmons, J. S.: Laboratory methods of the United States Army, Philadelphia, 1931, P. Blakiston's Son & Co., p. 364.

Method for neutralization of water

Neutralize enough water for the day's work. The bottle used for storing the neutral water should preferably be Pyrex or should be a bottle that has been used for no other purpose than to hold distilled water. No rubber connections should be used on the still. If distilled water is purchased in large quantities, it should be poured, not siphoned, into the bottle that is to hold the neutralized water.

Pick up a few hematoxylin crystals with clean dry forceps and place in a test tube. Rinse the tube with the water before testing because the tube might not be neutral. Pour approximately 5 ml. of the water to be neutralized into the test tube containing the hematoxylin crystals, shake, and note the color. If the water is neutral, it will become a pale pink within 10 sec. If the water is acid, it becomes yellow and remains yellow for longer than 5 min. If it is alkaline, it becomes reddish purple immediately or before 1 min. If the water is acid, as it usually is, 1% potassium carbonate is used as a neutralizing agent. If it is alkaline as it comes from the still, it should not be used. Add the neutralizing agent, a drop at a time, to the stock bottle of water to be neutralized, shaking after the addition of each drop or two and again testing the reaction. When the test water turns pink without a trace of yellow or blue in 10 sec., it is neutral and may be used. If too much potassium carbonate has been added, the water becomes alkaline. Pour out some of this alkaline water and add the slightly acid nonneutralized water. Proceed as before until the water is neutral.

Water handled in this manner gives excellent staining results with Giemsa or Wright stain. Staining characteristics of blood cells are altered with changes in the reaction of the water.

If the water is acid, erythrocytes are bright red orange instead of light yellowish orange. All eosin stains become very bright reddish orange. The eosinophils stand out particularly brilliantly. In a count of eosinophils alone it is advisable to use the acid distilled water. The nuclei of all cells will stain a pale sky blue. The cytoplasm of all lymphocytes and monocytes is very pale blue. The neutrophilic cells become extremely pale, the nuclei staining either pale blue or pale lavender, and the cytoplasm a definite pink. The age of the various neutrophils cannot be determined accurately because of indefinite staining of the nuclei, thus rendering a Schilling count practically an impossibility. Blood platelets are very pale blue. Malarial parasites lose much of their color, the red chromatin staining very pale and the blue cytoplasm being scarcely visible.

With alkaline water erythrocytes stain blue or green. A purple cast is imparted to all blues in the preparation. The cytoplasm of lymphocytes becomes gray or lavender and cloudy; the cytoplasm of monocytes is decidedly pinkish lavender or even purple; the cytoplasm of the neutrophils is deep lavender or almost red. Eosinophils show deep gray or blue granules. The granules of all neutrophils become very intensely overstained and appear more numerous and larger than normal. This makes the detection of toxic granules impossible. Chromatin granules of all nuclei assume the appearance of large black clumps. The identification of any cells having nucleoli becomes extremely difficult. Erythrocytes not only change in staining characteristics but also seem to have no center and frequently have horny edges so that all the cells look crenated. Most leukocytes look frayed at the edges. Malarial parasites stain particularly well, but if the water is too alkaline, it is impossible to differentiate the chromatin from the cytoplasm. Blood platelets swell and stain reddish lavender. One cannot identify them by their structure but only by their size and arrangement.

Another danger in staining blood films is in **using too little of the staining solution** or in **overwashing.** Water will decolorize the stain. Slides must be washed very slightly and then allowed to stand on end to dry. In staining, the slides should be placed on a staining bridge over a staining pan, and as much diluted stain as they can accommodate should be used. If slides are understained or overwashed, the true color will be lost, the nuclei and cytoplasm will blend instead of being distinct, and most of the cells will look very fragile. Understained cells look very much like cells stained with acid water.

Refer to Fig. 370, in which are shown the effects on blood cells of neutral water, alkaline water, acid water, and excess washing after staining.

Our routine is to neutralize about 2 L. water every day. The water should be tested each day since it sometimes becomes acid or alkaline upon standing.

Diluting the stain

Dilute only as much Giemsa stain as desired for the work at hand. Whereas stock Giemsa stain is stable and does not precipitate, diluted Giemsa stain will change within an hour or two.

Measure the neutral distilled water in the flask or cylinder used for the diluted stain. Add 1 drop Giemsa stain for each 1 ml. neutral distilled water, shaking very slightly during the addition of each drop.

Do not add all the Giemsa stain at once. The matter of shaking after adding each drop is important. Do not shake the flask too vigorously, as a precipitate may form. Shake only enough to ensure a good mixture of the stain and the water. The drop should be measured with a dropper, the opening of which is not too small.

If a drop bottle is used as a container for Giemsa stain, it must be prepared by allowing neutral distilled water to stand in contact with the glass for at least a week, changing the water each day. The bottle must be dried.

The Giemsa stain (stock) may then be placed in the drop bottle.

Fixing the film

Absolutely pure acetone-free methyl alcohol must be used.

The methyl alcohol should be kept in a Coplin jar and transferred at the end of the day to a bottle which should be kept tightly stoppered.

Fix the slides by placing them in the jar of methyl alcohol for 3-5 min. Remove and allow to dry in the air. The drying of the fixative should always be hastened as much as possible.

Staining the film

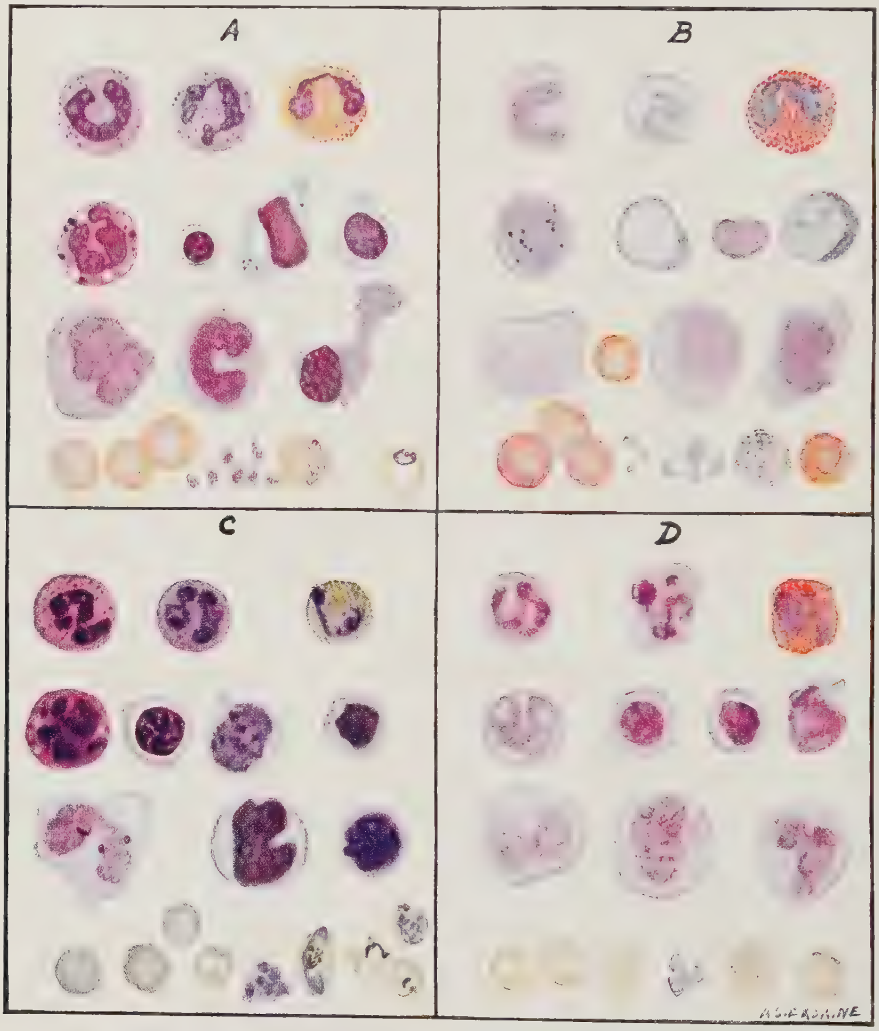
After fixation, place the slides on a staining bridge and flood them with diluted Giemsa stain. Allow the stain to remain on the slides for 30 min. or as long as 45 min. if desired. Pour off the staining mixture by gently tilting the slide and wash with neutral distilled water. The washing should not be lengthy, as too long washing will tend to decolorize the stain. Allow to dry in the air. If the slides contain an excess of blue, rewash them after they have dried and then allow them to dry in the air in a vertical position.

Rapid Giemsa method 1—McGee[129]

Stain the spread for ½ min. with a mixture of 1 part Giemsa stain to 2 parts acetone-free methyl alcohol. Mix 20 drops methyl alcohol and 10 drops Giemsa stain in a dry test tube and then flood the slide with the entire amount of this mixture for ½ min. Add an equal vol. neutral distilled water and allow the mixture to remain on the slide for 2 min. Flood the slide with neutral distilled water. Dry and examine. Use only for emergency work.

Rapid Giemsa method 2

Take equal parts of stock Giemsa stain and methyl alcohol. Stain 1 min. Differentiate until a red color appears. Dry between filter paper.



Copyright, 1935, by R. B. H. Gradwohl

Fig. 370. Blood cells and malarial parasites (Giemsa stain) with **A,** neutral water; **B,** acid water; **C,** alkaline water; and **D,** excess washing after staining. Top row: 2 neutrophils and 1 eosinophil. Second row: 1 basophil and 3 lymphocytes. Third row: monocytes. Fourth row: erythrocytes, blood platelets, and malarial parasites.

Changes in Giemsa staining to bring out special structures

For malaria: To stain blood spreads and thick drop preparations to search for malarial parasites, the water used should be slightly alkaline rather than neutral.

1. Add 1 drop 1% potassium carbonate solution to every 25 ml. neutral distilled water before adding the Giemsa stain. One drop Giemsa stain is added to each ml. water as usual (pH 7.0-7.2).

For eosinophils: To stain blood spreads and thick drop preparations to search for eosinophils, the distilled water used in diluting the Giemsa stain is allowed to remain slightly acid. The granules of the eosinophils with such staining solution stain decidedly eosin-pink.

For spirochetes: The stain should be alkalinized by adding 1 drop 1% potassium carbonate to each 10 ml. distilled water before adding the Giemsa stain.

1. Fix the slide, after drying, in methyl alcohol for 5 min. or in ethyl alcohol 20 min. Allow to dry.
2. Flood with diluted Giemsa stain, alkalinized, and steam for about 10 min. or stain cold for 24 hr.
3. Wash in running tap water for 3 min.

Spirochetes stain red. Other organisms are blue.

Wright stain

No fixative is necessary because Wright stain* contains methyl alcohol. Neutral distilled water is used. The timing of the stain must be determined when it is manufactured, but usually it is 1 min. staining and 4 min. after the water has been added.

1. Flood the slide with Wright stain and allow it to remain in contact with the film for 1 min.
2. Add neutral distilled water a drop at a time, not too slowly, until there is a mixture of equal parts of distilled water and stain.
3. Allow the mixture to remain for 4 min.
4. Flood with neutral distilled water to wash.
5. Dry in the air.

Modified technic for staining with Wright stain—Bercovitz

A slight modification of the preceding technic in making Wright stain has been suggested by Bercovitz.[130] The apparatus requires 2 staining jars with covers.

Procedure:

1. Fill one staining jar with pure methyl alcohol and the other with 30% Wright stain in distilled water.
2. Immerse the dry blood film in methyl alcohol at least 5 min.
3. Place the slide in the stain for 2 min. or longer.
4. Wash and dry as usual.

With this method there is no precipitate on the slide. The stain can be used repeatedly instead of only once. If the fluid levels of the reagents become low, add more solution to bring to the original volume. Do not discard the stain. No changes are noted even when slides are left for as long as 1 hr. in the methyl alcohol. The diluted stain can be used for several months.

May-Grünwald stain

The original communication on blood staining by the May-Grünwald method was published in *Centralblatt für innere Medizin.*[131] Granulations in the cells of sputum were demonstrated first by triacid staining as very fine red spots in the leukocytes.[132]

It was stated that these granulations were identical with the neutrophilic granulations described by Ehrlich.[133, 134]

May and Grünwald showed some interesting experiments with 4 kinds of eosin and 4 kinds of methylene blue directed toward the staining of cells and their granulations. They worked out a mixture of eosin and methylene blue directed toward the specific granulations in blood cells.

Stain and staining solution:

1. Mix 1 L. 1% eosin with 1 L. 1% methylene blue and after several days' standing filter by means of a suction pump. Wash the precipitate with cold distilled water until the filtrate is unstained.
2. Allow the sediment on the filter to dry. It consists, as seen under the oil immersion objective, of brown-red needle-form and rod-shaped crystals.

This material is slightly soluble in cold water, more so in warm water, better in ether, alcohol, acetone, etc., but best in methyl alcohol.

Make a saturated solution in methyl alcohol.

Procedure: The method of staining is the same as the Wright staining technic.

May-Grünwald-Giemsa or Wright-Giemsa staining of bone marrow and blood films

Spreads of bone marrow and blood may be stained by a combination of May-Grünwald-Giemsa or Wright-Giemsa stain. The advantages of such combined staining are that the nuclear pattern of cells is more distinctive and there is greater regularity of tinctorial reproduction from slide to slide. This combination staining shows the leptochromatic nuclear pattern of young leukocytes exceedingly well. Erythrocytes show a constancy of stain that permits evaluation of hemoglobin content.

Procedure:

1. Flood slide with Wright stain for 1½-2 min.
2. Add neutral distilled water or neutral buffer for 2 min.
3. Pour off and add dilute Giemsa stain for 7

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*The method for making Wright stain and staining solutions is given on p. 1139.

min. for blood spreads or 10 min. for bone marrow spreads.
4. Wash off and dry.

Dilute Giemsa stain is prepared by adding 2 drops stock Giemsa stain to each ml. neutral buffer or neutral distilled water. This diluted Giemsa stain may be kept for 4-6 hr. but should not be used beyond this period.

SPECIAL METHODS OF STAINING

Oxydase staining (to determine nature of various blood cells)

Oxydase staining is a useful method of determining whether there is a definite ferment in the blood which gives specific staining to myelogenous, monocytic, and lymphatic elements. A positive reaction to the peroxidase stain is seen in the neutrophilic cells, eosinophils, well-preserved basophils, juvenile and stab cells, myelocytes, promyelocytes, and adult monocytes. The reaction is negative in lymphocytes, some monocytes, erythroblasts, and all cells which have lost their granules. The method of choice is that advocated by Graham.[135]

Graham method

This method is recommended because the stained preparations are permanent.

Solutions:

1. Formalin-alcohol.

Alcohol, 95%	90	parts
Formalin, 40%	10	parts

2. Peroxidase reagent.

Alphanaphthol	1	part
Alcohol, 40%	95	parts
Hydrogen peroxide	0.2	part

This solution should be not more than 1 wk. old.

3. Pyronin solution.

Pyronin	0.1	part
Alcohol, 40%	95	parts
Anilin oil	4	parts

Shake vigorously but do not filter.

4. 0.5% aqueous methylene blue.

Methylene blue	0.5	part
Distilled water	100	parts

Filter. This stain gives better results when diluted 3:4 after making the 0.5% solution if National Aniline dye is used.

Procedure:

1. Keep the solutions in Coplin jars.
2. Make blood films in the usual manner.
3. After drying in the air, fix in formalin-alcohol 5 min. Rinse thoroughly in running tap water. Drain but do not dry. Dry back of slide.
4. Place in alphanaphthol solution (peroxidase reagent) 5 min.
5. Rinse thoroughly in running water. Drain but do not dry. Dry back of slide.
6. Stain in pyronin solution 10 min. Wash. Drain but do not dry.
7. Stain in methylene blue solution 12-20 sec.
8. Rinse. Let dry. Examine under low-power magnification with the condenser in place to make sure that the staining solutions reacted properly. Look for definite blue stain of nuclei. If not blue enough, stain again in the methylene blue.

Myelocytic elements show red granules. **Lymphocytic** elements stain blue. All nuclei stain blue. **Monocytes** are not characteristically stained, but the "pink dust" stains red, and appears as very fine stippling.

Before staining the patient's blood film, it is best to stain a film of freshly prepared normal blood to determine timing of stains and staining characteristics. The staining solutions seem to give better results if they are allowed to stand for 24 hr. before using although they may be used immediately after manufacture in an emergency.

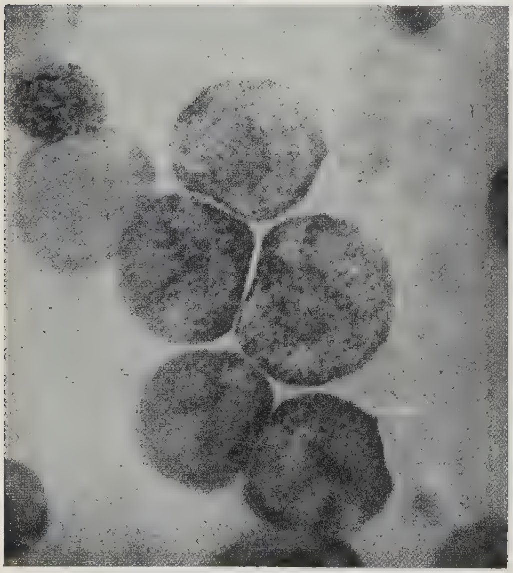

Fig. 371. Myelocytic leukemia. Blood spread, Graham oxydase stain, showing selective staining of granules. (×950.)

Oxydase reaction according to methods of Sato and Sekiya

Reagents:

1. Copper sulfate.
   Dissolve 0.5 gm. copper sulfate in 100 ml. distilled water.
2. Benzidine solution.
   Moisten 0.02 gm. benzidine, special for blood, with a few drops of water. Then grind. Add 200 ml. distilled water and filter. To filtrate add 0.25 ml. 3% hydrogen peroxide. Keep this solution in a brown bottle in the dark when not in use. It will remain good for a year although some precipitation occurs.
3. Safranin, 1% aqueous solution.

Procedure:

1. To a fresh, dry blood film apply the copper sulfate solution for 20 sec.
2. Drain off and apply the benzidine solution for 8 min.

3. Drain this off and add the safranin solution for 2 min.
4. Wash and dry.
5. Examine under oil immersion.

The peroxidase granules of myelocytes and segmented neutrophils have a deep green-blue color, whereas the nuclei are stained orange with the counterstain. Granules vary in number and size. Eosinophils show large heavy granules. Myelocytes show only a few granules; the younger they are, i.e., the nearer in age to the myeloblast, the fewer granules they have. The lymphocytes have no granules and the monocytes only a few near the nucleus. This reaction magnifies granules and serves to distinguish between the white cells of bone marrow origin and those of lymphocytic or reticuloendothelial origin.

To count oxydase stained spreads of blood

Use the 4-field meander method of counting (p. 1157). Count 200 cells, 50 in each field. Record these simply as oxydase-positive (myelocytic) elements, oxydase-negative (lymphocytic) elements, and oxydase-partially-positive (monocytic) elements. Report the percentage of each type of cell. For example:

		Normal
Myelocytic elements (oxydase-positive) . . .	85%	(63-77%)
Lymphocytic elements (oxydase-negative) . . .	13%	(21-30%)
Monocytic elements (oxydase-partially-positive)	2%	(4 to 8%)

In the preceding report, if this is leukemia, obviously it is myelocytic. The normal white cells of the differential count, when oxydase stained, occur in the following numbers: oxydase-positive, 63-77%; oxydase-negative, 21-30%; oxydase-partially-positive, 4-8%. Myeloblasts, promyelocytes, all myelocytes, juvenile and stab cells, segmented neutrophils, eosinophils, and basophils stain oxydase-positive except in unusual cases when they do not carry the oxydase ferment. Lymphoblasts, prolymphocytes, large and small lymphocytes, and plasma cells all carry no ferment and therefore are oxydase-negative. Monoblasts, promonocytes, and mature monocytes usually have a few extremely small oxydase-positive granules.

Demonstration of siderocytes or sideroblasts[136]

Siderocytes and sideroblasts are described on p. 1169. See also the discussion of sideroblastic anemias.

Materials:

1. Spreads of blood or bone marrow.
2. Formalin.
3. 2% aqueous solution of potassium ferrocyanide.
   2 gm. potassium ferrocyanide/100 ml. distilled water. This should be aged for several days.
4. 2% Hydrochloric acid.
   2 ml. concentrated hydrochloric acid added to distilled water and diluted to 100 ml. with distilled water.
5. Stock basic fuchsin solution.
   Dissolve 1 gm. basic fuchsin in 10 ml. absolute alcohol and add 90 ml. 5% aqueous phenol.
6. Working counterstain.
   Add 6 ml. stock basic fuchsin solution to 100 ml. distilled water.

Filter all solutions just prior to use.

Procedure:

1. Place blood or bone marrow films in a Coplin jar and cover the top of the jar with a piece of absorbent paper saturated with formalin.
2. Allow the slides to remain in this formalin vapor atmosphere for 30 min.
3. Stain 1 hr. in a mixture of equal parts of ferrocyanide and hydrochloric acid solutions.
4. Wash in tap water.
5. Counterstain with dilute basic fuchsin for 30-60 sec.
6. Remove excess basic fuchsin by washing in tap water and dipping in absolute ethyl alcohol.
7. Air dry.
8. Examine under oil immersion.

Siderocytes and sideroblasts will contain iron in the form of blue-staining granules within the cytoplasm.

Heinz body stain

Method I (unstable):

1. Mix a few drops citrated blood or blood taken in EDTA anticoagulant with a double volume of filtered 0.5% methyl violet in physiologic saline.
2. Mix gently and allow to stand at room temperature for 5-10 min.
3. Examine microscopically after placing a drop under a cover glass and then blotting off excess fluid and rimming with petroleum jelly.

Method II (permanent):

1. Mix a few drops citrated blood or blood taken in EDTA anticoagulant with a double vol. physiologic saline.
2. Centrifuge for 3 min. and pour off the supernatant fluid.
3. Resuspend the cells in filtered 0.5% methyl violet in physiologic saline.
4. Centrifuge for 3 min. and pour off the supernatant fluid.
5. Make films of the sediment, air dry, and mount with balsam (synthetic).

Alkaline phosphatase stain*

Method of Kaplow[137]

Reagents:

1. Fixative solution.

Formalin (36% to 39% formaldehyde)	10 ml.
Absolute methyl alcohol . . .	90 ml.

Store in the freezing unit of a refrigerator.

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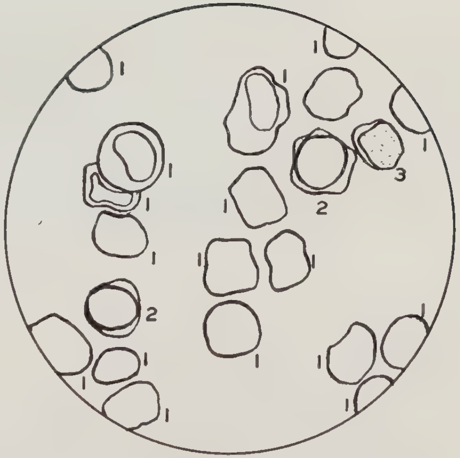
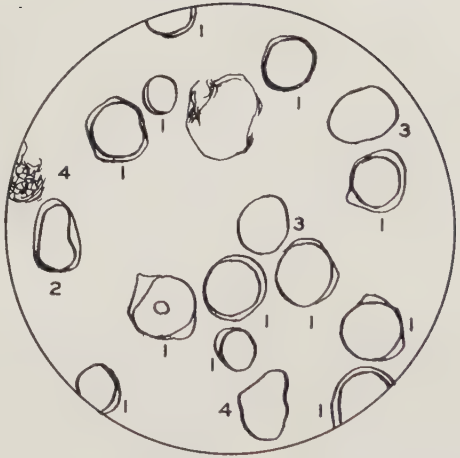
*From Cartwright, G. E.: Diagnostic laboratory hematology, New York, 1958, Grune & Stratton, Inc.



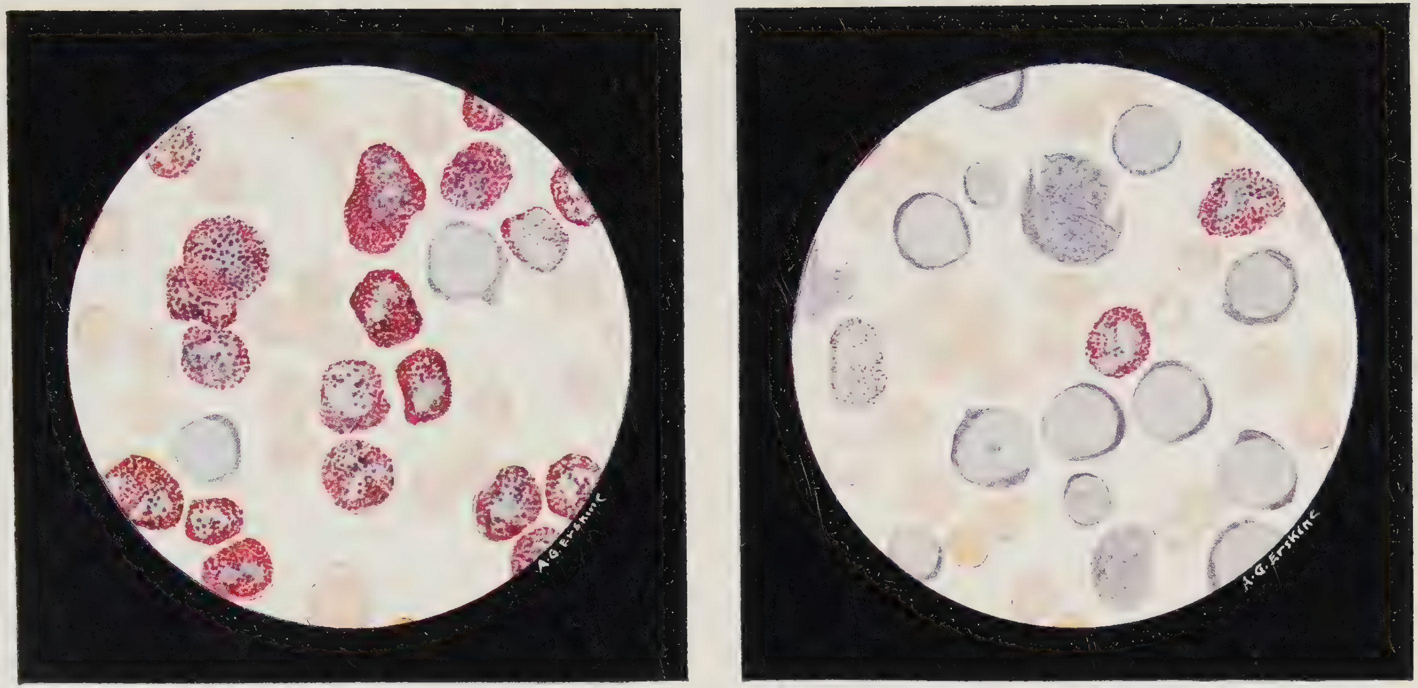
Myelocytic leukemia (×950)

1. Myelocytic cells
2. Lymphocytic cells
3. Monocytic cells



Lymphocytic leukemia (×950)

1. Lymphocytic cells
2. Monocytic cells
3. Myelocytic cells
4. Nuclear fragments


A.G. Erskine
A.G. Erskine

Myelocytic leukemia (×950)

Lymphocytic leukemia (×950)

Fig. 372. Graham oxydase stain.

2. 0.2M Propanediol stock solution.
   2-Amino-2-methyl-1, 3-propanediol* . . . . . 10.5 gm.
   Distilled water to make 500 ml.
   Store in a refrigerator.
3. 0.05M Propanediol buffer, pH 9.75.
   0.2M Stock solution of propanediol . . . . 25 ml.
   0.1N Hydrochloric acid . . . 5 ml.
   Distilled water to make 100 ml.
   Store in a refrigerator.
4. Substrate mixture, pH 9.5-9.6.
   Sodium alpha naphthyl acid phosphate† . . . . . . 35 mg.
   Fast blue RR‡ . . . . . . 35 mg.
   0.05M Propanediol buffer . . 35 ml.
   Filter and use at once.
5. Mayer aqueous hematoxylin:
   (a) Solution A.
      Hematein . . . . . . 1 gm.
      Ethyl alcohol, 90% . . . 50 ml.
   Incubate 24 hr. at 37° C.
   (b) Solution B.
      Potassium aluminum sulfate . . . . . . 50 gm.
      Distilled water to make 1 L.
   Mix solution A with solution B.
   Place the mixture in the sunlight for 3-4 wk.

Procedure:

1. Make a film of fresh blood and air dry.
2. Immerse the slide in the fixative for 30 sec. at 0° C.
3. Wash in running water for 10 sec.
4. Incubate in substrate mixture for 10 min. at room temperature.
5. Wash in running water for 10 sec.
6. Counterstain with Mayer hematoxylin for 3-4 min.
7. Wash in running water for 10 sec. and air dry.
8. Mount in synthetic balsam.

Interpretation: The granules of polymorphonuclear cells, stab and juvenile cells, and myelocytes are phosphatase positive and stain from pale brown to black, allowing grading from 0 to 4+, according to the number and color of the granules.

Grade 0 = colorless cytoplasm
Grade 1+ = pale brown cytoplasm and no granules
Grade 2+ = brown cytoplasm and occasional dark granules
Grade 3+ = dark granules
Grade 4+ = black granules

In the normal blood film, most neutrophils are Grade 0, a few are Grade 1+ to Grade 2+. In infections, trauma, infarction, severe hemorrhage, and myeloproliferative disorders, the alkaline phosphatase content of the granules is increased. In acute and chronic myelogenous leukemia, the alkaline phosphatase within the neutrophils is decreased. The alkaline phosphatase shows a significant rise (20 days) early in pregnancy and can therefore be used as a differential diagnostic test of ectopic pregnancy.[138]

Method using naphthol phosphate

The reagents for this test, which is satisfactory and rapid, can be obtained commercially.* Alkaline phosphatase deposits appear as purple spots.

SEDIMENTATION RATE OF ERYTHROCYTES†

John Hunter in 1797 is given the credit of first noting that the speed of sedimentation of the erythrocytes was increased in individuals suffering from inflammatory lesions and that this speed varied with the severity of the infection. Others (Nasse, 1836; Davy, 1839; Muller, 1844) noted the behavior of red cells but did not explain it. Interest was renewed by Fahraeus[139] in 1917. In 1920 Linzenmeier[140] worked out a technic. In this country, Friedlander,[141] Polak and Mazzola,[142] Baer and Reis[143] reported the value of the test in obstetrics and gynecology. Gruenfeld,[144] Cutler,[145] Bach and Hill,[146] Bannick,[147] and others have called attention to its usefulness in medical cases, particularly rheumatic fever and tuberculosis. Grodinsky, Lesser and Goldberger, and Smith have written of its advantages in general surgery.

Sedimentation of the erythrocytes is a complex biologic process, the basic mechanisms of which cannot be reduced to a common denominator. Fahraeus referred to the phenomenon as suspension instability, which is still a good description. The physicochemical forces which have been implicated are numerous and varied. The electrical charge of erythrocytes as related to proteins has been thought to play a role. Hoeber, Mond, and Ley note that hydrogen ion concentration influences the rate, with acidosis slowing and alkalosis accelerating it. The plasma proteins exert an influence on the sedimentation rate. Fibrinogen is perhaps the most readily demonstrable protein affecting sedimentation. In diseases associated with hypofibrinogenemia, such as liver disease, the rate is very slow and some observers have suggested that the sedimentation test be included in liver profile studies. The addition of fibrinogen to normal blood in vitro markedly accelerates settling of the erythrocytes. Meyers et al.[148] confirm this fact by in vitro experiments and call attention to α_2 globulin and gamma globulin as other protein fractions having a similar effect. These workers emphasize that the erythrocyte sedimentation rate alone "does not give any clue as to the level of any one of the responsible factors."* They offer 2 complex formulas based on plasma:cell ratio and plasma protein concentrations from which formulas they can predict the sedimentation rate. The concentration of proteins, perhaps qualitatively as well as quantitatively, in some unknown manner affects the aggregation of erythrocytes in the form of rouleaux or agglomeration.

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*Eastman Kodak Co., Rochester, N. Y.

†Dajac Laboratories, Leonminster, Mass.

‡General Dyestuffs Corp., Boston, Mass.

*Sigma Chemical Co., St. Louis, Mo.

†Part of this section is from a monograph by R. B. H. Gradwohl: The rediscovery of blood sedimentation test, Rev. de med. Trop. y Parasit. Bacteriol. 3:295, 1937.

Recently, the relationship of fibrinogen and gamma globulin to the sedimentation rate has been reinvestigated. Ruhenstroth-Bauer[149] came to the conclusion that "an increase in fibrinogen or gamma globulin is only coincidental to a high sedimentation rate and not its cause."† According to this author, the increased sedimentation rate is due to plasma proteins called **agglomerins** which resemble incomplete antibodies acting in a high molecular medium. Fibrinogen is only one fraction of the agglomerins. Antiinflammatory substances block agglomerins. Glucolipid fractions (receptors) in erythrocytes are able to combine with agglomerins, the production of which is stimulated by cellular proteins of homologous or heterologous nature. Every plasma contains an inhibitor substance which at body temperature is activated by a specific serum lipase.

The specific gravity of plasma does not play an important role because the addition of weak saline causes a diminution in sedimentation rate.

A purely physical factor in sedimentation rates can be readily demonstrated and is now common knowledge. If one sedimentation tube is set up perfectly perpendicularly and another tube of the same specimen is aligned at an angle, the latter will have a higher speed of sedimentation than the former. The temperature under which the test is performed affects the rate, the rate tending to be elevated as the temperature rises. These 2 factors are important controls in performing the test.

TECHNIC OF DETERMINING SEDIMENTATION RATE OF ERYTHROCYTES

Westergren method

Materials:

1. Westergren pipet, 200 mm. in length, 2.5 mm. diameter, graduated in mm.
2. Westergren block.
3. Tourniquet.
4. Sterile 2 ml. syringe.
5. Sterile 3.7% solution sodium citrate.
6. Alcohol, sterile sponges, and adhesive tape.
7. Watch glass.

Procedure:

1. Draw sodium citrate solution into the syringe to mark 0.4.
2. Apply the tourniquet to the patient's arm and locate the vein.
3. After disinfecting the skin, insert the needle into the vein and draw blood into the syringe up to mark 2 ml. Remove tourniquet and withdraw the needle.
4. Remove needle from syringe.
5. Draw back the plunger of the syringe and tilt back and forth several times to ensure a good mixture of the blood and citrate.
6. Eject contents of syringe into watch glass.
7. Draw the mixture into a Westergren pipet to mark 0 and place the pipet in a Westergren block in a vertical position.

The number of millimeters that the erythrocytes drop in the pipet in 1 hr. is the sedimentation rate.

Read the sedimentation of the erythrocytes in mm. at the end of 1 hr.

Normal: After 1 hr., 1-15 mm. for men; 0-20 mm. for women.

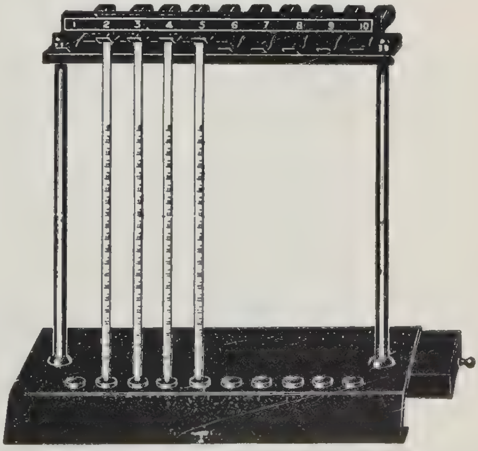

Fig. 373. Westergren sedimentation apparatus, improved Hellige model. (Courtesy Hellige, Long Island City, N. Y.)

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*From Meyers, A. J., et al.: Blood 8:893, 1953.

†From Ruhenstroth-Bauer, G.: Brit. M. J. 5249: 1804, 1961.

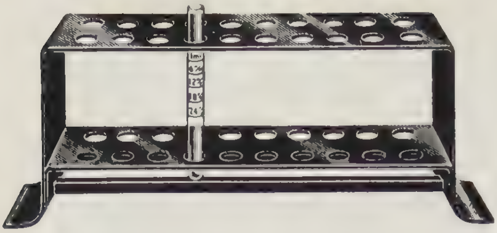

Fig. 374. Rack for use with Linzenmeier or Cutler sedimentation tubes. (Courtesy Hellige, Long Island City, N. Y.)

Linzenmeier method

Materials: Tubes 5 mm. in diameter, 65 mm. in height, with two marks, one at the level of 1 ml. and the other 18 mm. below this. Other equipment same as Westergren method.

Procedure:

1. Draw 0.2 ml. 3.7% sodium citrate solution and 0.8 ml. blood into the syringe and mix.
2. Pour the mixture into the tube up to 1 ml. mark.
3. Allow to stand in an upright position until the red blood corpuscles have settled to the mark 18 mm.
4. Note the time for this to take place. Record in minutes.

Normal: Healthy men, 350-600 min.; healthy women, 300-600 min.; menstruation, 600 minutes. Anything under 200 min. is pathologic.

Graphic or Cutler method

Materials:

1. Tubes 5 ml. capacity, graduated in 0.1 ml., each 0.1 ml. being 1 mm. in height.
2. Sterile 5 ml. syringe.
3. Freshly prepared 3% sodium citrate solution, sterile.
4. Other equipment same as Linzenmeier method.
5. Sedimentation report charts, which are available commercially.*

Procedure:

1. Draw into the syringe 0.5 ml. sodium citrate solution.
2. Draw blood to mark 5 ml.
3. Mix as in Linzenmeier method.
4. Pour contents into sedimentation tube.
5. Stopper with a paraffin-coated cork.
6. Allow tubes to stand 10 hr. but observe every 5 min. for 1 hr.
7. Draw curve on sedimentation chart.

The sedimentation value, determined by the sedimentation of the red cells in the first hour, depends

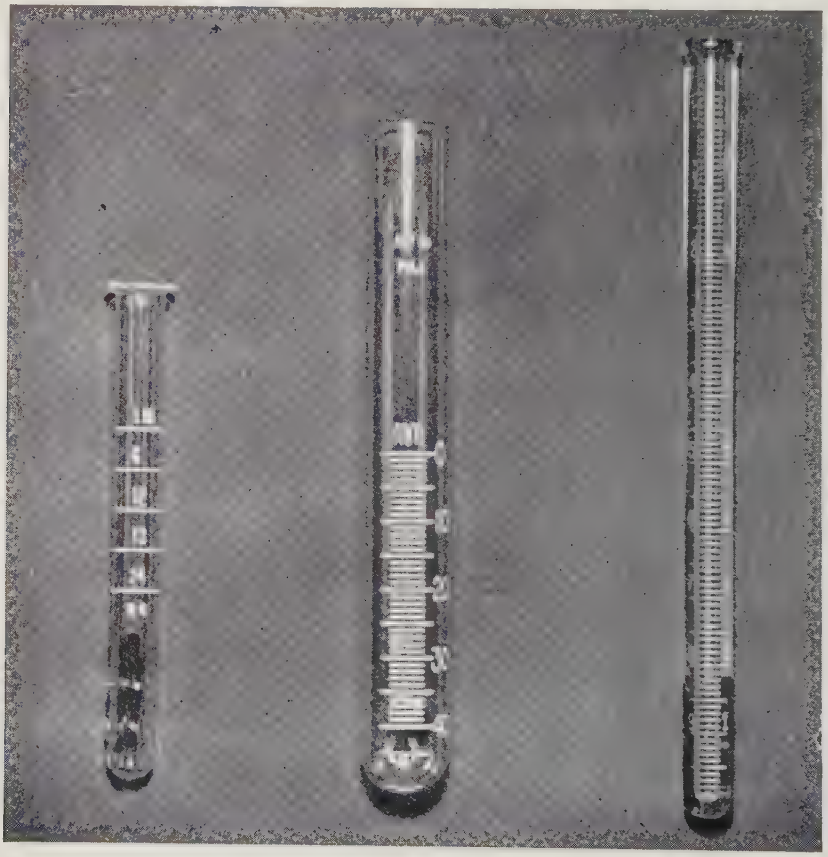

Fig. 375. Linzenmeier, Cutler, and Wintrobe tubes.

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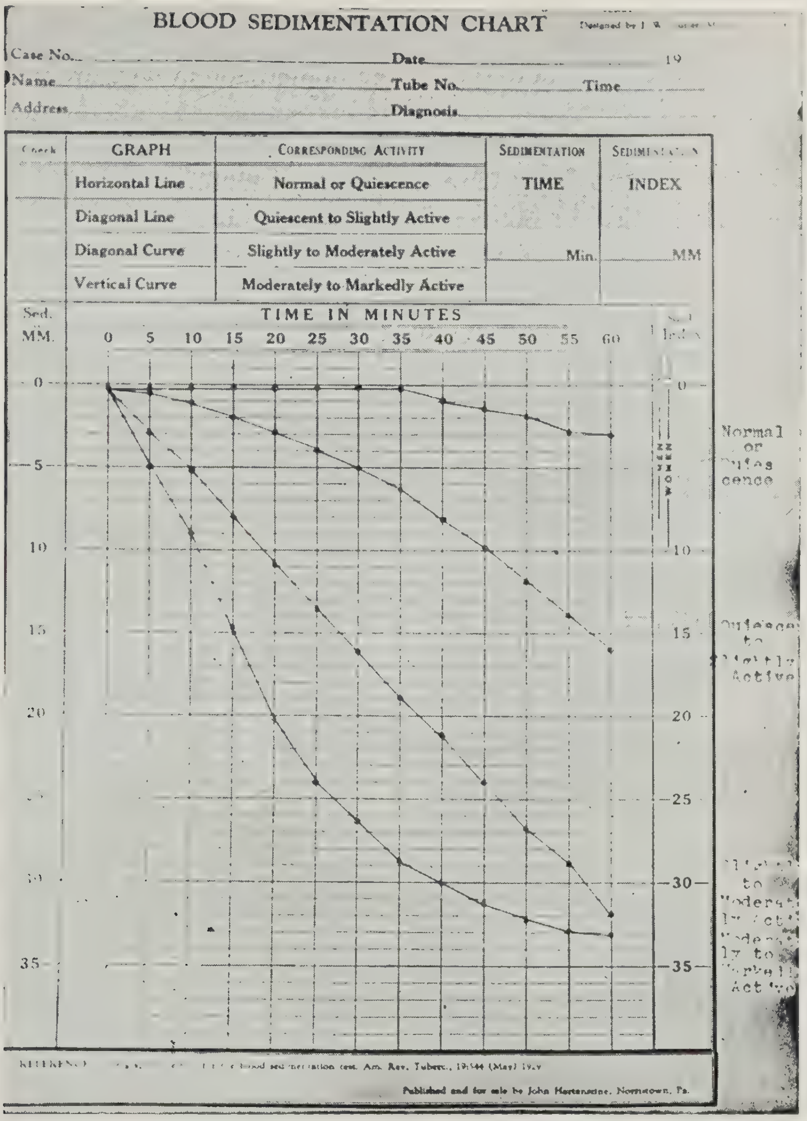
*Charles M. Berkemeyer, Sellersville, Pa.; Clay-Adams Co., New York, N. Y.; Aloe Scientific, St. Louis, Mo.; etc.



BLOOD SEDIMENTATION CHART

Case No. Date 19

Name Tube No. Time

Address Diagnosis

Check	GRAPH	CORRESPONDING ACTIVITY	SEDIMENTATION TIME	SEDIMENTATION INDEX
	Horizontal Line	Normal or Quiescence		
	Diagonal Line	Quiescent to Slightly Active		
	Diagonal Curve	Slightly to Moderately Active	Min.	MM
	Vertical Curve	Moderately to Markedly Active		

Published and for sale by John Hortenstine, Norristown, Pa.

TIME IN MINUTES
Sed. MM.
0
5
10
15
20
25
30
35
40
45
50
55
60
0
5
10
15
20
25
30
35
Normal or Quiescence
Quiescent to Slightly Active
Slightly to Moderately Active
Moderately to Markedly Active

Fig. 376. Cutler chart.

upon the sedimentation graph, the sedimentation index, and the sedimentation time. There are 4 graphs, 2 straight lines, and 2 curves. These are called horizontal line, diagonal line, diagonal curve, and vertical curve.

Sedimentation index is the total sedimentation of the red cells at the end of 60 min. expressed in mm.

Normal: Men, varies from 0-8 mm., average 3-4; healthy women, 0-10 mm., average 5-6; in menstruation as high as 12 mm.

Wintrobe-Landsberg method

Materials:

1. Wintrobe tubes and venous blood collected in 3:2 oxalate mixture (p. 1100).

   Place the filled Wintrobe tube (Fig. 375) in an exactly vertical position at room temperature and observe the point on the mm. scale to which the corpuscles fall during 1 hr.

Normal: The normal average sedimentation in 1 hr. by this method is 3.7 mm. for healthy men and 9.6 mm. for women, with a maximal range from 0.9 mm. for men and 0-20 mm. for women.

Comment: For many years it was recommended that the sedimentation rate be corrected for the volume of packed red cells. Wintrobe now believes that this adjustment is an unnecessary refinement of the procedure.[150]

Landau method[151] (modification of Linzenmeier-Raunert method)

1. Before using, clean the sedimentation pipet well with alcohol and ether and dry thoroughly in an oven or other warm place. If it is not completely dry, sodium citrate solu-

tion should be drawn up before the blood test is taken.

2. Insert capillary pipet into a precision syringe.
3. Draw up 5% sodium citrate solution by turning the top screw to the left until the upper meniscus of the solution has reached the lower mark **A.**
4. Lance finger tip or ear lobe of the patient deeply enough to get an even flow of blood without squeezing.
5. Draw up the blood until the height of the liquid reaches the upper meniscus of the upper graduation **B** and try to avoid the formation of air bubbles rising in the blood solution. If bubbles should form during the test, they can easily be expelled by means of the top screw. The suction of the blood sample can easily be performed with one hand by holding the syringe with the capillary pipet in dagger fashion with the middle, fourth, and small fingers and manipulating the top screw with thumb and index finger.
6. Clean the tip of the capillary pipet with ether and continue to draw up the mixture into the bulb (by turning top screw to the left) until the lower meniscus of the blood column ends just a few mm. below the lower opening of the bulb. (Do not draw blood into bulb completely.) The pipet may then be carefully shaken.
7. Force the blood up and down twice very slowly by turning screw to the left or right, respectively, in order to obtain a better mixture in the bulb. Force the blood gradually back into the capillary tube, close lower opening with thumb, and hold pipet firmly while turning the syringe carefully, exerting a slight pull in order to lift out the capillary pipet which is then placed into the metal stand.

The blood mixture may hang at any place in the tube but should not touch the bottom. Citrated blood should always be drawn slowly into the capillary tube; otherwise part of it will cling to the walls and air bubbles will form. If air bubbles have formed in the blood column, turn syringe upside down at an angle and force out the air bubbles by turning the screw to the right. This will push the blood column toward the opening of the pipet and thereby expel the air bubbles without the loss of much blood.

The instrument consists of a metal stand which holds either 6 or 12 capillary pipets, 12 cm. long with an inside diameter of 1 mm. Each pipet is provided with 2 graduation marks, one at 12.5 and the other at 62.5 mm. above the tip. The pipets are held in place by strong bottom springs which press the pipet against corresponding cavities provided in the screws, which are fitted into the upper part of the metal stand directly vertical above the bottom springs.

To insert the pipets into the stand, press down with the tip of the pipet against the bottom spring, and while holding it down, insert the upper part of the pipet into the cavity by gradually releasing the pressure on the spring. In the upper part of the pipet is a small bulb similar to the bulb in an ordinary blood pipet.

The precision syringe consists of a metal barrel

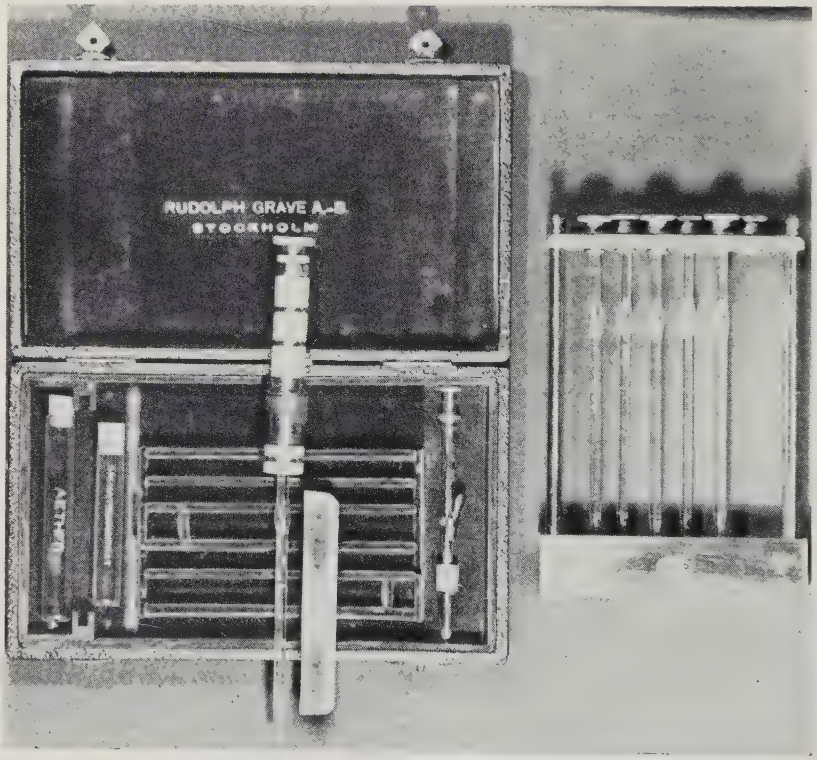


Fig. 377. Landau modification of Linzenmeier-Raunert blood sedimentation outfit.*

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*May be procured from Clay-Adams Co., Inc., New York, N. Y.

with ground-in plunger which is adjusted by means of a screw. The lower part of the barrel has a perforated rubber stopper fitted inside into which a capillary pipet can be inserted. By adjusting the bottom screw, the size of the perforation of the stopper can be increased or decreased. Near the bottom, the barrel of the syringe has a fine opening in the side which ordinarily is covered with a piece of rubber tubing. Before inserting the capillary pipet, which already has been filled with citrated blood, into the syringe, it is necessary to roll the pointed part of the rubber tubing upward with the thumb and thus expose the side opening of the metal cylinder ordinarily covered by the tubing. By covering the tip of the capillary pipet with the finger, the pipet may be inserted into the syringe as far as it will go, and then the rubber tubing is released. In keeping the side opening of the pipet exposed, an excess of pressure in the syringe can be avoided which would tend to expel the blood. In the event that the capillary pipet has a very small outside diameter, the rubber stopper in the syringe may have to be exchanged for one with a smaller opening. If the plunger should fit too tightly, a drop of oil, dropped into the cylinder's upper part, will usually make it slide smoothly again.

The metal housing with a capacity of 6 capillary pipets is protected by a velvet-lined case which also holds glass vials for ether and 5% sodium citrate solution and automatic blood lancet, rule, rubber tubing, compartment for the precision syringe, and room for cotton and compressors. The stand, together with glass capillary pipets, can be placed in horizontal position in the case.

Results: Readings are to be made in mm. after 1 hr. The original length of the blood column is 62.5 mm.

Because of the length of the bulb, when the precision syringe is in use, only very little citrated blood will cling to the walls of the capillary pipet; thus the definite length of the column would deviate only one or a few mm. at the most from the original length, and this small difference would be of no practical importance. One can also determine the length of the column as approximately 60 mm.

Normal: For children under 2 yr. of age and for men, 1-5 mm./hr. For children over 2 yr. of age and for women, 1-8 mm./hr. Slightly increased values are 6-14 mm. and 9-14 mm., respectively. Fairly high values are 15-25 mm. High values are over 25 mm.

The Landau modification of the Linzenmeier-Raunert method of sedimentation is especially useful in pediatrics since it does not entail venipuncture. Venipuncture in children is often attended with difficulties; therefore, any method that avoids it is to be recommended.

Summary of the pertinent facts on sedimentation test

Normal:

Westergren:
  Men: 0-15 mm. after 1 hr.
  Women: 0-20 mm. after 1 hr.

Linzenmeier:
  Men: 350-600 min.
  Women: 350-600 min.
  Under 200 min. is pathologic.

Cutler:
  Men: 0-8 mm. after 1 hr. (3-4 mm. in 1 hr., average)
  Women: 0-10 mm. after 1 hr. (5-6 mm. in 1 hr., average)

Wintrobe and Landsberg:
  Men: 0-9 mm. in 1 hr. (3.7 mm., average)
  Women: 0-20 mm. in 1 hr. (9.6 mm., average)

Landau:
  Men: 1-6 mm. in 1 hr.
  Women: 1-9 mm. in 1 hr.

Comments on sedimentation rate determination

Peightal[152] very properly believes that the test is not a specific diagnostic sign for any disease but indicates the presence or absence of an inflammatory process. In some conditions it may be used in part to estimate the severity of an infection.

Landau,[151] in discussing the usefulness of microsedimentation rate in pediatrics, emphasizes its employment in arthritic and tuberculous conditions. In a number of cases, the test was useful in children, bedfast for a long time, whose primary illnesses during their stays at the hospital were followed by regularly made microsedimentation tests. In such cases an increase in sedimentation rate occurred as the symptom of a complication several days before a rise in temperature occurred. Usually the microsedimentation rate decreased much later than the temperature fell, sometimes reaching the desired value only several weeks after the temperature had become normal. Westergren considers pronounced sedimentation to be characteristic of acute polyarthritis. High values in light cases of acute polyarthritis are important as an indicator of the intensity of the process of the disease. Not until sedimentation has reached normal values after repeated tests can one regard the infectious process as having run its course.

Since apparently rheumatic illnesses are very common in Gothenburg, Sweden, Landau had numerous opportunities of testing this method in such cases. He found it quite useful in following the progress of acute polyarthritis, in acute endocarditis, pericarditis, and myocarditis, and in chorea. He has found this test very helpful in facing this important question: When, after the temperature has subsided to normal, can one venture to pronounce the endocarditic process ended in acute endocarditis? In other words, when does the endocarditis end and the vitium, the resultant condition, begin? This also raises the important question of the continued therapeutic regimen, particularly physical therapy, as well as the prognosis for cardiac function. Landau states that, in addition to cases in which the diagnosis is doubtful, microsedimentation has proved to be of extraordinary value as an indicator of disease, being generally far more sensitive than the body temperature. In routine treatment of acute polyarthritic and cardiac diseases at the Gothenburg Hospital for children, it was his custom not to release patients from bed until microsedimentation had shown normal values after repeated tests, irrespective of whether the body temperature had become normal. We do not agree that such rigidity in management is necessarily wise and that return to activity can be judged by the sedimentation test **alone.** The **total** clinical picture must be taken into account for such decisions.

In a study of 25 cases of chorea it was found that when chorea had existed as the only rheumatic condition, when polyarthritis or endocarditis had preceded it so long before that chorea could not be considered as having arisen in direct connection with these diseases, or when complicating commonplace infection could be precluded, repeated microsedimentation tests showed low values within the limits of normal. On the other hand, in cases in which the chorea arose at the same time as, or in direct connection with, polyarthritis, endocarditis, or pericarditis, microsedimentation rate was increased, and the sedimentation curve seemed to be influenced by the other diseases. It was upon this finding that Landau based his agreement with Vollenberg's hypothesis of chorea as a metarheumatic disease or as an intoxication of the cerebral cortex.

The blood sedimentation rate has been used extensively in following patients with pulmonary tuberculosis as a gauge of activity and response to therapy. In tuberculosis of the bronchial lymph nodes in children during periods of activity the blood sedimentation rate is elevated. Westergren states that blood sedimentation in bronchial lymph node tuberculosis in children after 1 hr. is 50 mm. during the period of activity. It then sinks rapidly and after 2 mo. is around 10 mm. Dehoff found that in active tuberculosis of the hilus the blood sedimentation rate with the Linzenmeier tube was such that 280 min. were required for the blood plasma to reach mark 18 mm. Rókay, using the Westergren method, found the blood sedimentation values as given in Table 238A in cases of hilar node tuberculosis.

Table 238A. *Westergren sedimentation rates in tuberculosis of hilar lymph nodes**

Sedimentation rate (mm.)	*No. of cases*	*Class I*	*Class II*	*Class III*
0— 3	2	–	2	–
4— 6	7	2	4	1
7— 12	16	6	4	6
13— 17	1	–	1	–
18— 25	3	1	2	–
26— 37	3	–	2	1
38— 50	1	–	–	1
51— 70	–	–	–	–
71— 89	–	–	–	–
90—120	1	–	–	1

*Class I, slightly enlarged nodes; Class II, glands size of hazelnut; Class III, nodes size of hen egg.

In Rókay's second table the activity of the tuberculous process is compared with the blood sedimentation (Table 238B).

Table 238B. *Relation of sedimentation rate to activity of tuberculosis*

Sedimentation rate (mm.)	*No. of cases*	*Activity*		*Prognosis*		
				Good	*Dubious*	*Bad*
		0	+			
0— 3	2	2	–	1	1	–
4— 6	7	5	2	7	–	–
7— 12	16	10	6	9	7	–
13— 17	1	–	1	–	1	–
18— 25	3	–	3	–	3	–
26— 37	3	–	3	–	1	2
38— 50	1	–	1	–	–	1
51— 70	–	–	–	–	–	–
71— 89	–	–	–	–	–	–
90—120	1		1	–		1

Blood sedimentation rate in children with polycythemia is very high, whereas in adults it tends to be low. According to Schönberger,[153] in myxedema the blood sedimentation rate is enormously accelerated, but glandular therapy may return it to normal very rapidly.

In arthritis in adults the sedimentation rate is definitely increased in the infectious or atrophic rheumatic and rheumatoid type and is normal in the noninfectious or hypertrophic degenerative osteoarthritic type.

In psychoneurotic patients with unusual symptoms in whom no organic lesion may be found, repeatedly normal sedimentation rates aid in excluding organic disease.

The sedimentation rate is retarded in asthma, hay fever, and urticaria and other allergic states.

In pertussis, with the sedimentation rate normal and a high lymphocyte count, we have an unusual condition, namely, a normal rate in a febrile disease. In all other febrile diseases, it is apparently accelerated.

It must be borne in mind that the sedimentation rate and the hemogram are both independent of the fever curve. They both may be negative or positive with and without fever, but they parallel temperature reactions only in the climax of severe inflammatory processes. Expressed in other terms, the hemogram reaction is exceedingly useful in acute infections, whereas the sedimentation rate is important in the more chronic conditions.

The sedimentation rate has been found useful in following the course of coronary occlusion in adults. The rate tends to be high early and increases as the myocardial infarction spreads. Decline in the rate occurs when the process of infarction ceases. In other cardiac diseases in adults not associated with infarction, the sedimentation rate tends to be normal.

In summary, the sedimentation rate finds a wide field of clinical applicability.[154] It is not a specific diagnostic test although it may influence differential diagnosis. Its greatest usefulness is in following the course of diseases known to have increased sedimentation rates. As an adjunct to its reflection of the progress of such diseases, the sedimentation rate acts as a yardstick of the efficacy of treatment. Although single determinations have value, **changes** in multiple determinations in a given case are of even greater significance in the evaluation of a patient.

Methodology in sedimentation rate determinations deserves some comment. The various types of apparatus and the dissimilarity of reporting results attest to the interest manifested in this procedure. In general, we feel that the test has been made unnecessarily complicated, considering its clinical usefulness. Any method with 1 reading in an hour giving the amount of settling of the erythrocytes appears quite adequate. Those methods requiring graphs of various types appear to us as unnecessary refinements.

The anticoagulant used is important. Sequestrene and heparin are not suitable unless combined with 3.8% citrate.[155] The Westergren method may lead to erroneous results because of the dilution of the blood, whereas the Wintrobe technic may also lead to wrong results in certain viscous sera (multiple myeloma) because of the absence of dilution. Dawson[156] attempted a compromise by using solid sequestrene and 1 part 3.8% citrate to 4 parts blood.

Greisheimer et al.[157] attempted to draw conclusions about the average relationship existing between the Cutler, the Linzenmeier, and the Westergren methods. They found that a sedimentation of 1 hr. for normal subjects seems to be reasonably concordant for the 3 methods despite the wide differences in tube width, anticoagulant concentration, and length of fluid column, although the differences between the means for the 3 methods are significant statistically. The average sedimentation at 1 hr. for women is approximately double that for men. They showed by tables the various indices and differences between the 3 methods and attempted to establish some ratios between them. Their tables demonstrate that the regression lines for predicting the most likely value to be expected by any one test when that by another is known are of three distinct types. There is a rectilinear relationship between the Cutler index and the Linzenmeier index. The linear regression differences between Westergren index and Cutler or Linzenmeier index appear to be somewhat like saturation curves. It was impossible to fit this into a systematic scheme by any type of mathematical equation. The relationships between the various indices and Linzenmeier time values may be suitably portrayed by sections of hyperbolae. Individual tables have been given by them for each independent variable of clinical interest. The dispersals of individual cases about the lines of average relationship, although not studied in detail, are clearly greater than those ascribable to errors inherent in the technics. It is obvious that sedimentation measures for human blood are, in part, specific for the technic employed. It would be advantageous, therefore, to have a standardized method for blood sedimentation for clinical work, but as yet this has not been done. Therefore, in estimating the importance of the sedimentation rate in any individual case, we must be familiar with the sedimentation rate in such cases, with the method used, and with the normal value for that method.

Watkins[158] emphasized the fact that variations in room temperature markedly affect the sedimentation rate. Sedimentation rate is retarded in a cooler room. Table 239 gives comparisons on stored blood and blood rechecked after 18 hours.

Table 239. *Relationship of sedimentation rate to temperature*

Case	*Room temperature reading*	*After 18 hr. at 10° C.*
1	24 mm.	(1 hr.) 3 mm. (2 hr.) 5 mm. (3 hr.) 9 mm.
2*	31 mm.	15 mm.
3†	27 mm.	25 mm.
4	24 mm.	17 mm.
5‡	(25° C.) 12 mm.	(37° C.) 20 mm.

*Hemolytic anemia.

†Pregnancy in latter months.

‡The variation between 25° and 37° C. in the sedimentation rate of erythrocytes is exactly 8 mm. There is a change from a normal or approximately normal to a definitely increased sedimentation rate of erythrocytes.

In blood sedimentation tests, either an electric light bulb or preferably a standard bacteriologic incubator can be used quite satisfactorily to supply heat. Racks containing the blood can be placed near the lamp or in the incubator. A thermometer must be placed near the blood so that records of the temperature can be made quickly and accurately. Watkins preferred the small Cutler tube for this work because (1) the smaller tube affords less surface area exposed to the reducing of temperature, (2) smaller amounts of blood can be used, (3) it is less cumbersome in handling and placing in the incubator, and (4) it maintains constant temperature. A few years ago Manley[159] devised temperature correction charts (normograms) for some of the methods.

It is important to hold the blood near body temperature until the test can be recorded. Chilling the blood before making the test will definitely alter the final results.

The **influence of anemia on the blood sedimentation rate** has been studied by many hematologists. Certain authors have suggested a correction of the sedimentation rate that takes into consideration the number of erythrocytes, the blood volume, the corpuscular volume, and the average diameter of the erythrocytes or their hemoglobin content.

According to Adura,[160] the influence of the erythrocytes on the sedimentation rate, if present at all, is very small in vivo. Therefore, for actual practice, consideration of the erythrocytes is unnecessary. The essential factors in sedimentation of erythrocytes are the physicochemical and electric alterations of the plasma. Specific weight of the erythrocytes is not to be considered a major factor. Adura points out that the sedimentation rates differ greatly in cattle and horses although the specific gravity is identical. Comparative studies made on hypochromic and hyperchromic erythrocytes showed that hemoglobin content does not materially influence the sedimentation rate. It can be shown also that the average diameter of the erythrocytes is of no significance in this respect. Adura pointed out that when erythrocytes from pregnant women or from patients with infectious diseases (accelerated sedimentation) were washed and then placed in the plasma of normal persons a normal sedimentation rate was obtained. On the other hand, when the washed erythrocytes of healthy individuals were placed in plasma of pregnant women, the sedimentation rate was greatly accelerated.

We are in agreement with Adura that these experiments prove that the plasma is the regulating factor in blood sedimentation, and that correction of the sedimentation rate on the basis of factors concerning the erythrocytes may even diminish the value of the reaction since thereby an extrinsic and inconstant factor is introduced which unnecessarily complicates the otherwise easy test.

The comments in the preceding two paragraphs have appeared in previous editions of this text. Wintrobe, who has been a strong advocate of correction of the sedimentation rate for anemia, now has abandoned such corrections as an unnecessary refinement of a crude test. Note of his present stand under the Wintrobe technic has been made, and the correction chart which formerly accompanied his technic has been eliminated. Meyers et al.[148] tried to devise some means of adjusting all sedimentation rates to a standard cell volume, and as a result of their studies cast "serious doubt on the validity of any such correction charts."[148]

Some years ago, Phear[161] reviewed this problem and found an increase in the sedimentation rate with increasing cell size but found no correlation between mean corpuscular hemoglobin concentration and the sedimentation rate.

DIFFERENTIAL COUNT

The differential count is also known as the qualitative leukocyte count. Its purpose is to determine the proportion of leukocytes of different types. This was done first in films by Einhorn[162] in 1884, followed by Kronig[163] in

1899, on spinal fluid, by Widal and Ravaut[164, 165] in 1900 (also on spinal fluid); by Simpson[166] in 1906; by Sheaff[167] in 1912; by Schilling[168] in 1913; by Nieuwenhuijse[169] and Johns[170] in 1914. Up to the announcement of Schilling in 1913, the method was known as the Ehrlich count for the reason that the types of cells described and counted were those noted by Ehrlich in his classic studies on the tinctorial qualities of various blood cells. It was Arneth who attempted to show that we might divide the immature cells into various forms and thus secure much more information as to the severity of infection present. His studies and suggestions, while essentially important, were not adopted because of the intricate details necessary to make a so-called Arneth count. Furthermore, according to Arneth's theory, leukocytes leave the bone marrow as young forms with simple indented nuclei, and these cells become changed into mature polymorphonuclear leukocytes in the circulating blood.

Schilling denies that the presence of the increased percentage of immature cell forms in the blood necessarily means an increased production of leukocytes by the bone marrow. He believes that the picture may be changed by immature cells which enter the blood being injured by bacterial or other toxins so that they fail to undergo differentiation. For instance, in typhoid fever, the decrease in the total number of white blood cells in the blood and increase in the number of immature neutrophils Schilling explains as being due, not to excessive destruction of white cells in the circulation with regenerative overactivity of the bone marrow, but rather to actual depression of marrow function, with a discharge into the blood of injured young neutrophils which do not become differentiated into the more segmented bodies. Although Schilling's work was first published in 1913, it seems to have escaped general attention, at least in the United States and practically so on the Continent, until a translation of his classic book from the German was published in 1929.[171] His remarkably simple classification of immature leukocytes, together with an interpretative formula, has been followed by a rather wholesale departure from the other methods of differential blood counting so long in vogue.

Since the popularization of the Schilling method, other methods of counting have been introduced, notably that by Cooke and Ponder in which 5 different types of cells are classified, depending upon the number of nuclear lobes. Any increase in the number of cells of the first 2 forms, i.e., the youngest type, is interpreted as a shift to the left. Then again we find the simplified Arneth count of Ponder and Krumbhaar in which neutrophils are divided into 3 classes. Finally, Farley and others have offered the filament and nonfilament neutrophil count as a method of choice in the diagnosis and prognosis of infection.

We do not find, however, that any count other than the Schilling gives as complete an understanding of the effects of infection in regard to diagnosis and prognosis.

Tabulation of differential count

The leukocytic blood picture is obtained by the differential count which consists in counting 100, 200, or 500 stained leukocytic cells, arranging them in the classification to which they belong, and reporting the percentage of each cell tabulated.

The 4-field meander method is recommended. Place a drop of oil in the middle third of each half of the film, as shown in Fig. 378. Twenty-five or 50 cells are usually counted in each field. The count is made by beginning the enumeration of the cells at the edge of the film, working toward the center (by moving the mechanical stage), going not quite to the center, and then working back to

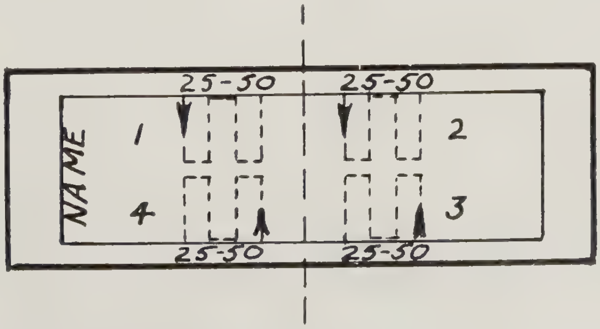

Fig. 378. "Four-field meander" method of differential counting.

BELL SIGNALS TOTAL OF EACH 100
TOTAL

Fig. 379. Marbel calculator for tabulating differential counts.

the edge and then toward the center until the desired number (25 or 50) of cells has been tabulated. After counting either 25 or 50 cells, as the case may be, shift to the next indicated field and begin the enumeration there. Continue in this manner until either 100 or 200 cells have been counted. It is our custom to count 50 cells in each field and divide the total of each cell counted by 2 to obtain the percentage of that type of cell.

Do not count only the edges since the large cells tend to accumulate there, and since the lymphocytes congregate in the center, an inaccurate count is obtained if only the center is counted. By using the 4-field meander method of counting a more accurate count is obtained.

Now construct a hemogram by recording the percentage of each cell counted. Record any abnormalities of the erythrocytes, the presence of blood parasites, and unusual staining such as toxic granules. All reports on the examination of differential counts must include an enumeration of abnormalities encountered in the erythrocytic cells. When the differential count is completed, a survey examination of the erythrocytes and platelets should be made so as not to overlook any pathologic findings.

There are various methods of mechanically computing the different cells in the differential count. The Marbel calculator is one type of instrument (Fig. 379).

Normal %	10	20	30	40	50	60	70	80	90	100	10	20	30	40	50	60	70	80	90		200 Cells Counted	%
0.5 (0-1) Basophil																/					1	½
3 (2-4) Eosinophil		/							/				/			/	/				5	2½
0 Myelocytes																						
0.5 (0-1) Juvenile				/																	1	½
4 (3-5) Stabs	/	/		/				//		/			/			/	/		/		10	5
63 (58-66) Segments	/ 卌	卌	// 卌	// 卌	卌 卌	卌	// 卌	// 卌	////	卌	/ 卌	// 卌	//	// 卌	//// 卌	///	////	//// 卌	////	//// 卌	123	61½
23 (21-30) Lymphocytes	///	//	///	/		卌	///	/	///	////	//	//	////	///	/	///	///	/	////	/	49	24½
6 (4-8) Monocytes		/							//		//	/	//			/	/		/		11	5½
																					200	100

REMARKS:
White cells: 12,000
Atypical cells (describe):
Hematocrit (cell vol.%):
Mean corpuscular volume:
Mean corpuscular hemoglobin:
Mean corpuscular hemoglobin concentration:

Red cells: 4,500,000
Hemoglobin: 13.26 gm./100 ml. Spencer, 84.9%
Polychromasia: +
Normoblasts:
Megaloblasts:
Anisocytosis: slight
Special forms:
Parasites:

Color index:
Volume index:
Saturation index:
Basophilic punctation:
Poikilocytosis:
Platelets:

Fig. 380. Hemogram chart including Schilling classification.

Record of erythrocytic cells in differential count

In a differential count all changes in erythrocytes are noted, and a written report is made of the findings. Observe first for anisocytosis, then for poikilocytosis, polychromasia, basophilic punctation, and then for any other abnormalities of the cells. The actual numbers of all types of nucleated red cells must be given per 100 leukocytes, together with a record of karyorrhexis and mitosis. Report anisocytosis, poikilocytosis, polychromasia, and basophilic punctation as marked, moderate, or slight. Report in % the number of the various types of nucleated reds (polychromatic normoblasts, orthochromatic normoblasts, etc.) per 100 leukocytes on the slide. If Howell-Jolly bodies, Cabot ring bodies, or any other changes are noted in the erythrocytes, such findings must be recorded in the written report which is presented to the physician. In addition to the degree of basophilic punctation, it is well to report the size of the dots as fine, medium, or coarse.

The erythrocytes are classified according to the size of the majority of erythrocytes and their pigment content into 7 groups as follows:

1. Normocytic normochromic, normal-sized erythrocytes with normal pigment content
2. Normocytic hypochromic, normal-sized erythrocytes with decreased pigment content
3. Microcytic hypochromic, small erythrocytes with decreased pigment content
4. Microcytic normochromic, small erythrocytes with normal pigment content
5. Macrocytic normochromic, large erythrocytes with normal pigment content
6. Macrocytic hypochromic, large erythrocytes with decreased pigment content
7. Macrocytic hyperchromic, large erythrocytes with apparent increased pigment content (often called macrocytic normochromic)

If the majority of erythrocytes are normal in size, but there are scattered macrocytes, this would be reported as normocytic with a tendency to enlargement of the cells.

The description of the red cell series is based on the acceptance of 2 separate types of maturation of red cells as shown in the outline that follows.

There is a normal form of erythropoiesis and a megaloblastic form of erythropoiesis.

Normal erythropoiesis

Normoblast (rubriblast) → Basophilic normoblast (prorubricyte) →
Polychromatic normoblast (rubricyte) → Orthochromatic normoblast (metarubricyte) →
Reticulocyte (only special stain) → Erythrocyte (normal and abnormal forms)

Megaloblastic erythropoiesis

Promegaloblast (rubriblast, pernicious anemia type) → Basophilic megaloblast (prorubricyte, pernicious anemia type) →
Polychromatic megaloblast (rubricyte, pernicious anemia type) → Orthochromatic megaloblast (metarubricyte, pernicious anemia type) →
Megalocyte

Megaloblastic erythropoiesis, as will be shown later, is the result of vitamin B_{12} deficiency.

The description of these cells refers to cells stained with Wright or Giemsa stain. For a discussion of physiology and origin of these cells see preceding chapters.

Chapter 62

Morphology of cells in peripheral blood

JOHN D. BAUER

VARIOUS FORMS OF ERYTHROCYTES

Acanthrocytes: Acanthrocytes are thorny appearing crenated spherical erythrocytes which are usually artifacts produced in the process of making blood films or in hypotonic solutions, representing one of the stages of spherocyte formation. In contrast to the artificially produced forms, there is a genetically determined abnormality characterized by the presence of acanthroctyes (acanthrocytosis) which is associated with pigmentary degeneration of the retina.[172]

Anisocytosis: The term anisocytosis describes a variation in size of red blood cells beyond the normal limits.

Anulocytes: Anulocytes are poorly hemoglobinized cells with only a peripheral ring of hemoglobin.

Basophilic stippling: See discussion of polychromatic erythrocytes.

Burr cells[173]**:** Burr cells are erythrocytes which have long, sharp spinelike processes. They have been described in the blood in uremia and in such diseases of the stomach as carcinoma and peptic ulcer. Crenated red blood cells are characterized by short, blunt projections.

Cabot ring bodies: Cabot ring bodies are very delicate circular, figure-of-eight or loop-shaped threadlike bodies frequently seen in polychromatic or punctated erythrocytes and sometimes seen in erythrocytes with nuclear spheres. They are seen in very severe anemias. Cabot[174] described these bodies as red and blue staining circular and twisted bodies within basophilic, polychromatic, and, infrequently, orthochromatic erythrocytes. They were brought to his attention by one of his associates in a case of lead poisoning and thereafter were observed by him in the blood of patients suffering from pernicious anemia, lead poisoning, and lymphocytic leukemia. There has been much discussion in the literature as to the nature of these ring bodies. Two groups represent the division of opinion on the subject—those who advocate that the ring bodies are remnants of the nuclear membrane and those who claim that they are artifacts. Cabot's own words in his original paper are as follows: "There is no reason to believe them a mark of cellular degeneration . . . that they have some connection with the nucleus formerly possessed by the cell that contains them seems to me not unlikely . . . their shapes correspond for the most part very well with those of nuclear figures . . . the best hypothesis, therefore, that I can suggest to account for these appearances is that they represent nuclear remains . . . the significance of their red color with Wright's, a stain which never stains any nucleus red, is not clear to me . . . have these figures any practical significance? Not so far as I know."*

The following investigators believed the rings to be identical with the nuclear membrane: Schleip,[175, 176] Pappenheim,[177-179] Sluka,[180] Alder,[181] Ferrata,[182, 183] Ferrata and Viglioli,[184] Juspa,[185] and Jordan, Kindred, and Beams.[186] Demel[187] proposed that the Cabot rings are artifacts. Naegeli[188] took a middle ground by stating that Cabot rings are generally considered nuclear membrane remnants, absent in erythroblasts and the blood-forming organs; they are a pathologic phenomenon of nuclear dissolution. Schilling[189] believed that it had not been proved that the Cabot-Schleip rings are nuclear remnants. In another publication,[190] he recorded seeing Cabot rings in normal erythroblasts.

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*From Cabot, R. C.: J. M. Res. 9:15, 1903.

Schleicher[191] has rather definitely proved that the Cabot ring bodies are denatured protein configurations and are actually laboratory products. He showed that Cabot ring bodies may be demonstrated by special methods and that they are neither nuclear remnants nor are they identical with the nuclear membrane. They are laboratory creations, the expression of cellular degeneration induced by hemolytic agents; i.e., they are the expression of aggregated and denatured colloid protein. He was able to produce these bodies experimentally. This work was carried out with egg whites on slides. He experimentally produced replicas of Cabot ring bodies in normal mature erythrocytes but not in normal erythroblasts. The creation of the bodies depends upon the physicochemical state of the lipoproteid colloids at the various maturation stages. For fuller information on the method of the experimental investigations, see his original article.

Elliptocytes: Elliptocytosis or ovalocytosis represents a congenital anomaly of red blood cells in which over 25% of the red blood cells are oval. In the camel this feature is physiologic, but in man normally only 1-15% of the red blood cells may be elliptocytes. Under pathologic conditions (macrocytic and microcytic anemias) the incidence may rise to 25%. The defect is mendelian dominant and is not sex-linked. Ovalocytosis is confined to the mature red blood cell and does not involve the precursor forms. In most cases elliptocytosis is asymptomatic, but occasionally it is associated with a hemolytic anemia (see discussion of hemolytic anemias, elliptocytosis).

Erythrocytes, crenated: Crenation occurs when red blood cells are exposed to hypotonic or hypertonic solutions. Crenated red blood cells are characterized by short, blunt, spikelike projections. Their presence, for instance, in cerebral spinal fluid indicates that the blood in the spinal fluid is not due to faulty technic (bloody tap).

Erythrocytes (normocytes): Erythrocytes or red blood cells are found normally in the circulating blood. They are biconcave disks without nuclei, 7.5-8μ in diameter and 1.7-2.2μ thick. Normal red blood cells are orthochromatic. The orthochromatic cells are the mature cells. Polychromatic cells are younger cells and may be found in the proportion of 2-10 faintly polychromatic cells/1000 orthochromatic erythrocytes in normal blood films. Red blood cells stain a uniform dull orange when orthochromatic or blue-gray when polychromatic. Any variation from this type of cell is pathologic or the result of poor technic.

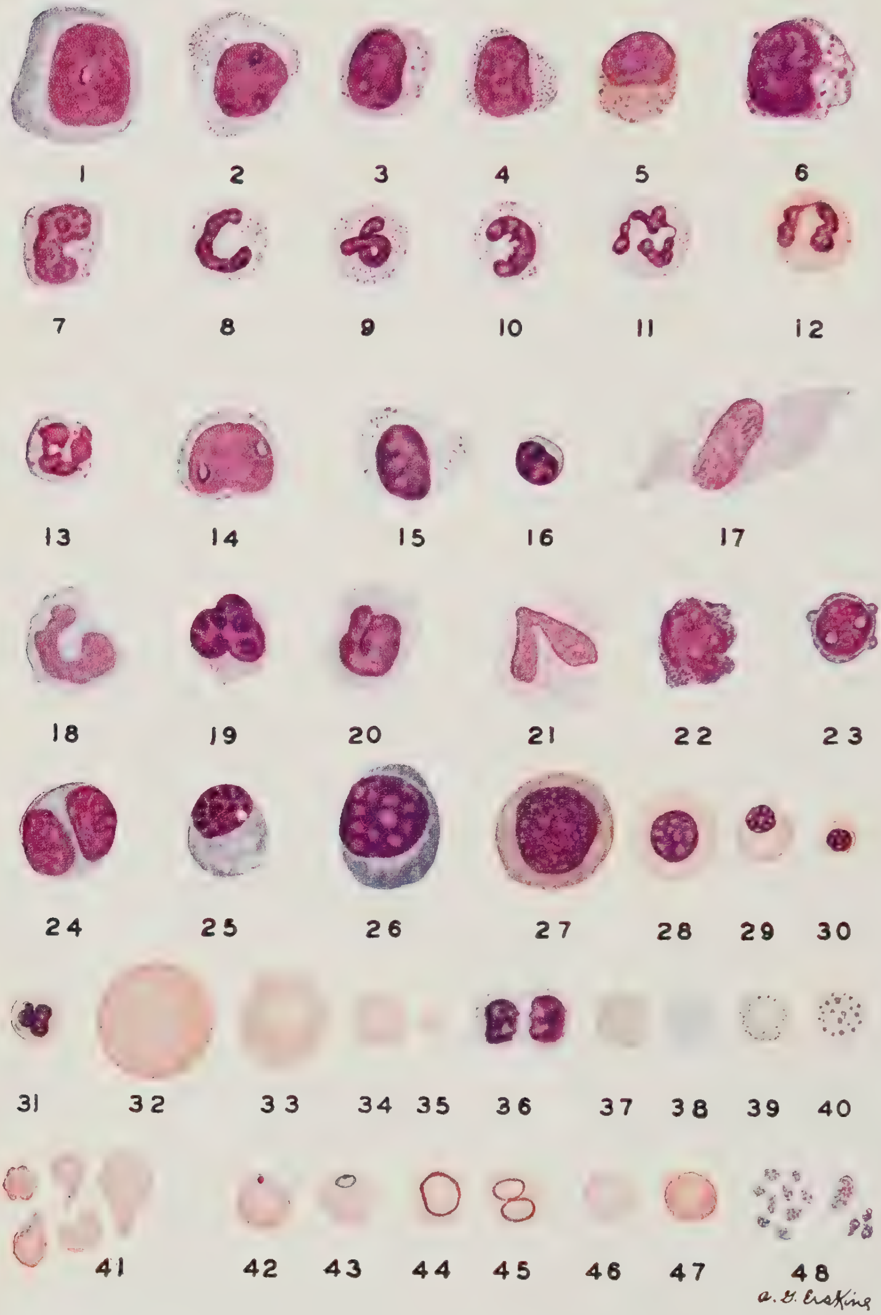
Erythrocontes: Erythrocontes are fine faintly red-staining rods seen in erythrocytes, especially in the stippled cells of pernicious anemia and other conditions.

Erythrocytic crescent: Crescent bodies are delicate almost colorless veil-like disks, 40-50μ in size, with pink crescents in the margin. They might be mistaken for parasites but are nonspecific general indicators of anemia. They often result from faulty technic.

Erythrocytic shadows: Erythrocytic shadows are dehemoglobinized erythrocytes which occur in making the preparation. They are caused by lack of resistance of the erythrocytes. They stain pale pink.

Heinz bodies (inner bodies): Inner bodies are bodies in erythrocytes demonstrated with supravital staining methods. They include **Ehrlich's hemoglobinemic inner bodies. Schmauch's inner bodies** in cats are partially identical with Ehrlich's inner bodies and partially with nuclear spheres.

Heinz bodies are seen in unstained blood preparations but cannot be seen in blood films stained with Wright or Giemsa stain. These bodies stain with supravital dyes and are usually found in mature or old erythrocytes (not in reticulocytes or polychromatic cells). They occur after splenectomy and in drug-induced hemolytic anemia in which the red cells show a defect of glucose 6-phosphate dehydrogenase.[192] This enzyme maintains glutathione in a reduced state, thus protecting hemoglobin from the toxic action of drugs. Butler[193] described a Heinz body formation test using acetylphenylhydrazine which distinguishes drug-sensitive erythrocytes from nonsensitive ones. Drug-sensitive red blood cells form Heinz bodies more readily and in greater number per red blood cell. Heinz body anemia has been described in premature and full-term newborn infants. In the group of Heinz body anemias of the newborn may fall the hemolytic anemias of the newborn due to excessive doses of vitamin K to the infant as well as so-called nonspherocytic congenital hemolytic anemia and perhaps some of the cases of unexplained neonatal hyperbilirubinemia.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A. G. Erskine

Fig. 381. Blood cells. (Giemsa stain.)

(See explanation of figures on opposite page.)

There is a voluminous literature dealing with the nature of the Heinz body.[194] Electron-microscopic observations suggest that changes in the stromatin[195] might be responsible for Heinz body production, whereas other work implicates methemoglobin as at least initiating the formation of these bodies.

Hemoglobin-H inclusions: These inclusions, which are sometimes found in thalassemia, are not seen in unstained cells but can be visualized with supravital stains (see discussion of abnormal hemoglobins).

Howell-Jolly bodies: Howell-Jolly bodies are peculiar spherical nuclear remnants seen in erythrocytes. They may graduate down to the size of a needle point. They are present after splenectomy and in severe anemias. They stain deep purple.

Karyogenic metachromasia (**Ferrata**)**:** Karyogenic metachromasia is a faintly wine red discoloration of erythrocytes due to the presence of fully dissolved chromatin particles. It is not found normally.

Karyorrhexis: Karyorrhexis of immature erythrocytes appears as bizarre cloverleaf nuclear shapes. This is indicative of beginning disintegration of the nucleus and is, of course, a pathologic finding. **Karyolysis** is dissolution of the nucleus of immature erythrocytes.

Leptocytes: The term is synonymous with target cells, platycytes, and Mexican hat cells (see discussion of target cells).

Macrocytes: Macrocytes are erythrocytes that are larger than normal cells, ranging up to 14μ in diameter. They contain no nuclei and are not found normally in the circulating

Fig. 381. Blood cells. (Giemsa stain.)

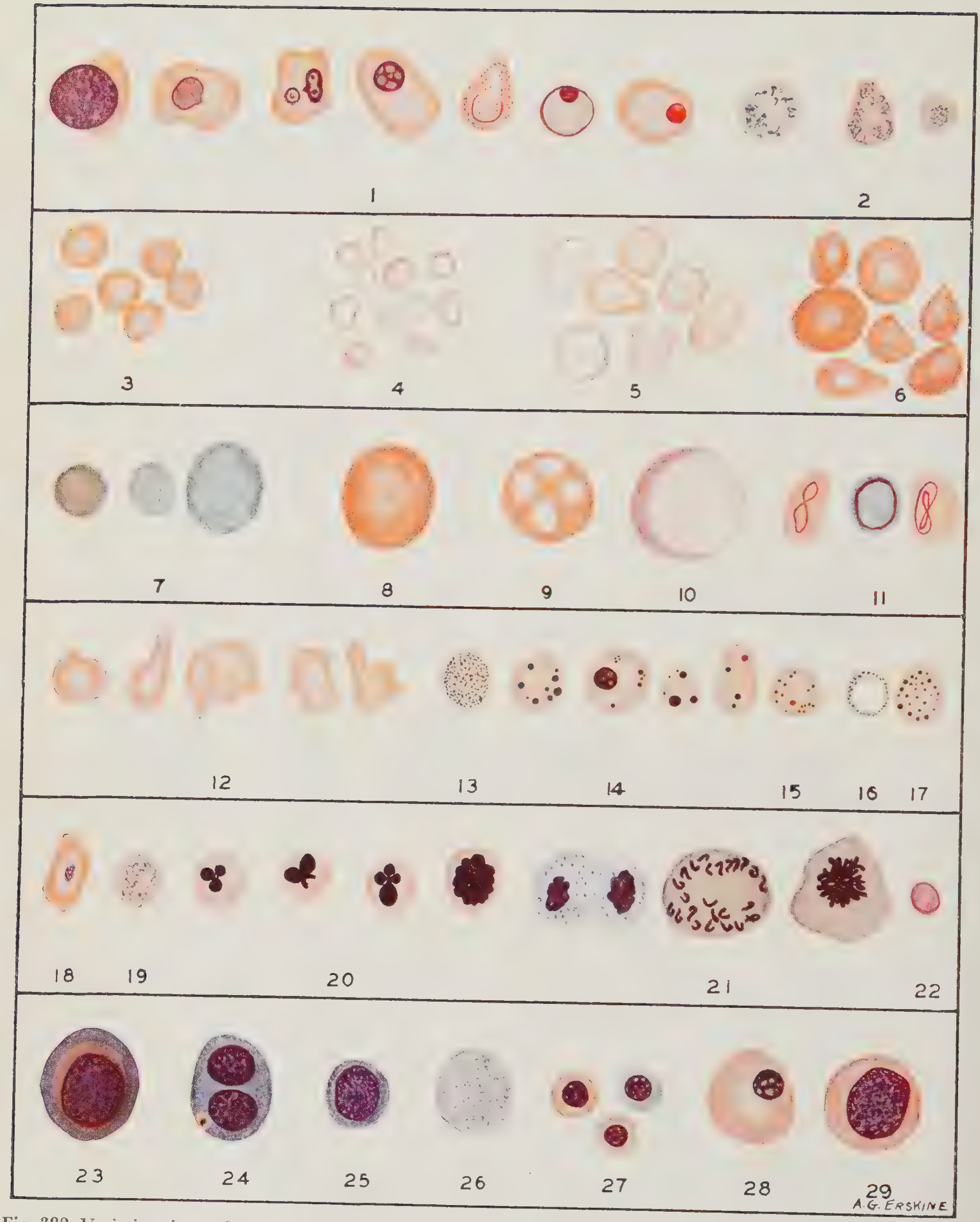
1. Myeloblast.
2. Promyelocyte.
3. Neutrophilic myelocyte (form usually seen in myelocytic leukemia).
4. Neutrophilic myelocyte (form usually seen in infections).
5. Eosinophilic myelocyte.
6. Basophilic myelocyte.
7. Juvenile (neutrophilic).
8. "Stab" (neutrophilic).
9. Twisted "stab" (neutrophilic).
10. Degenerated "stab" (neutrophilic).
11. Segmented neutrophil.
12. Eosinophil.
13. Basophil.
14. Lymphoblast.
15. Large lymphocyte.
16. Small lymphocyte.
17. Reticuloendothelial cell.
18. Monocyte (formerly called "transitional").
19. Monocyte.
20. Monocyte.
21. Endothelial element.
22. Atypical promyelocyte.
23. Micromyeloblast.
24. Myeloblast with 2 nuclei, no mitosis.
25. Plasma cell.
26. Large plasma cell.
27. Polychromatic megaloblast.
28. Orthochromatic macroblast.
29. Orthochromatic normoblast.
30. Orthochromatic microblast.
31. Normoblast with nucleus showing karyorrhexis.
32. Hyperchromic megalocyte.
33. Macrocyte.
34. Normocyte.
35. Microcyte.

32-35. Example of anisocytosis

36. Erythroblast with mitotic nucleus.
37. Faintly polychromatic erythrocyte.
38. Polychromatic erythrocyte.
39. Basophilic punctation, fine.
40. Basophilic punctation, coarse.
41. Poikilocytes.
42. Marginal granule.
43. Ring form of malarial parasite.

44, 45. Cabot ring bodies.

46. Erythrocyte showing hypochromia.
47. Hyperchromic erythrocyte.
48. Blood platelets.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A.G. ERSKINE

Fig. 382. Variations in erythrocytic cells.

(See explanation of figures on opposite page.)

blood. They may be polychromatic or orthochromatic.

Marginal corpuscles: Marginal corpuscles are very small (less than $\frac{1}{4}\mu$) single or double granules in an erythrocyte in an extremely eccentric or peripheral position. They are very easily detachable, are seen in only a few erythrocytes in the film, stain red, and are seen particularly in the regenerative anemias.

Megaloblasts: Megaloblasts are never found in the circulating blood in health. The cell is slightly oval and the cytoplasm contains hemoglobin in varying quantities. The cells, therefore, may be basophilic, polychromatic, or orthochromatic; i.e., they stain a deep blue, a bluish orange, or the orange of the mature erythrocyte. The different types of megaloblasts vary in size as follows: basophilic megaloblasts, 15-22μ; polychromatic megaloblasts, 10-18μ; orthochromatic megaloblasts, 8-15μ.

By phase microscopy and electron microscopy numerous mitochondria are seen within this cell. The so-called intermediate megaloblast will be considered in the discussion of megaloblastic anemias. Suffice it to say that its nuclear chromatin pattern is coarser than that of a megaloblast of pernicious anemia. The cytoplasm lacks evidence of hemoglobinization. Its appearance is due to the addition of iron deficiency to the already existing vitamin B_{12} and folic acid deficiencies.

The nucleus is oval or round, with a fine chromatin structure. There are no granules.

Megalocytes: Megalocytes are giant erythrocytic cells (15-18 or 20μ in diameter), are usually oval, and are never found normally in the circulating blood. They have no nuclei.

Metachromatic substance (substantia metachromatica): Metachromatic substances are fine globules within erythrocytes. They are best demonstrated with supravital stain. They accumulate in one place but may be distributed, often showing lively molecular motility in the darkfield. According to Pappenheim they are lipoid segments. They probably bear some relationship to the archoplasmatic plasmasomes.

Microblasts: Microblasts are small nucleated erythrocytes which may be either orthochromatic or polychromatic. Do not confuse them with lymphocytes. The nuclear structure will make the differentiation. They are never found normally in the circulating blood.

Microcytes: Microcytes are very small red blood corpuscles (5μ in diameter), not found normally in the circulating blood. True microcytes are usually hypochromic.

Normoblasts: Normoblasts are nucleated red blood cells. They are never found normally in the circulating blood. The cytoplasm may be orthochromatic, polychromatic, or

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Fig. 382. Variations in erythrocytic cells.

1. Ferrata's views on formation of Cabot ring bodies.
2. Reticulocytes—supravital stain.
3. Normochromic normocytic cells.
4. Hypochromic microcytic cells.
5. Hypochromic macrocytic cells.
6. "Hyperchromic" macrocytic cells.
7. Polychromatic erythrocytes.
8. Orthochromatic megalocytes—hyperchromic.
9. Orthochromatic megalocyte showing endoglobular degeneration.
10. Erythrocytic crescent.
11. Cabot ring bodies.
12. Poikilocytes.
13. Basophilic punctation, medium.
14. Howell-Jolly bodies, or nuclear spheres, some in a nucleated red cell.
15. Basophilic punctation in a cell with a marginal granule.
16. Basophilic punctation, fine.
17. Basophilic punctation, coarse.
18. Blood platelet on an erythrocyte.
19. Supravital stain of an erythrocyte, showing reticulum and a hemoglobinemic inner body.
20. Nucleated erythrocytes showing karyorrhexis.
21. Erythroblasts in mitosis.
22. Karyogenic metachromasia.
23. Polychromatic megaloblast.
24. Polychromatic megaloblast with 2 nuclei, without evidence of mitosis.
25. Polychromatic macroblast.
26. Megalocyte with erythrocontes.
27. Normoblasts, 1 of which is orthochromatic and 2 polychromatic.
28. Giant normoblast.
29. Orthochromatic megaloblast.

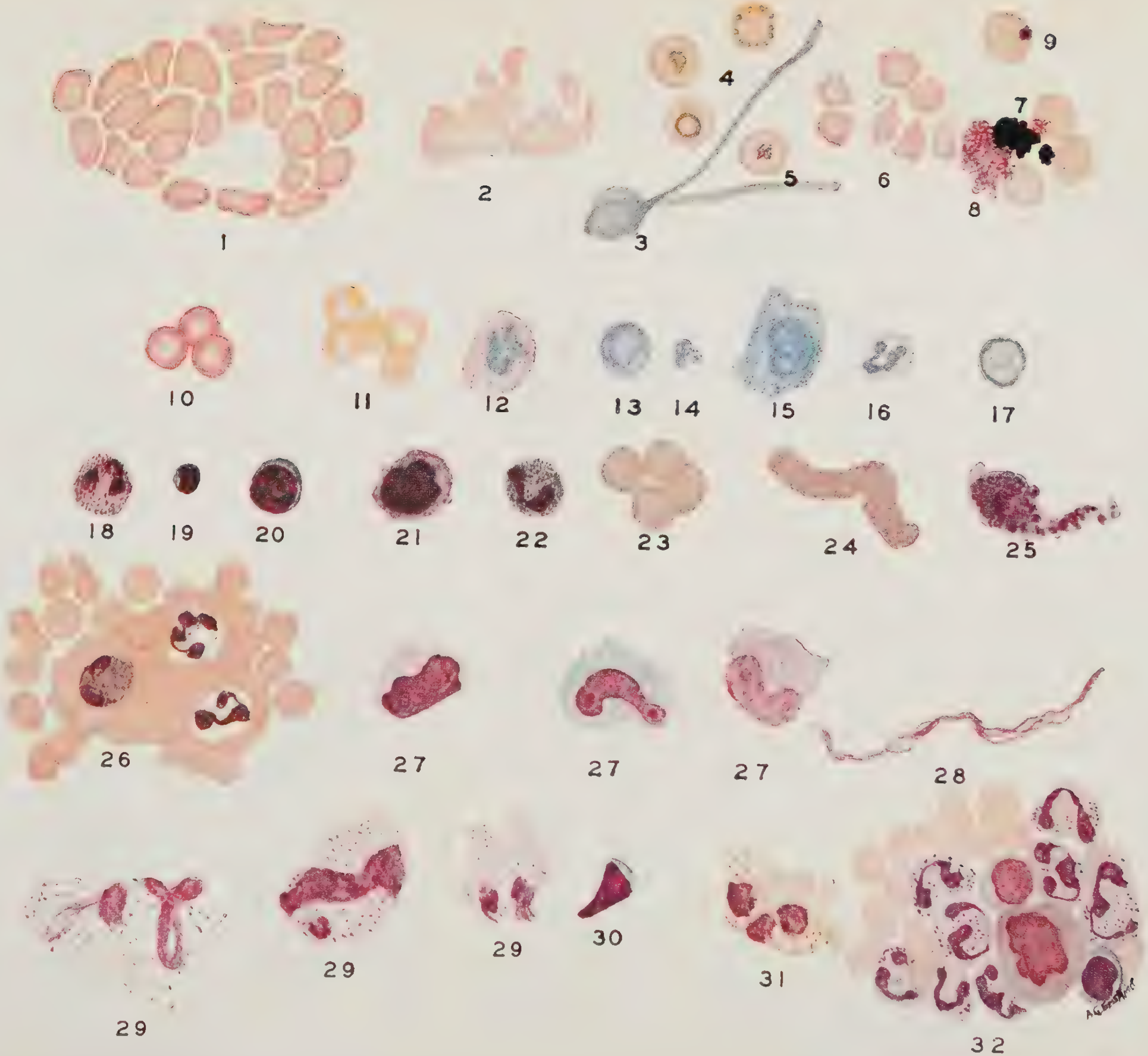
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Fig. 383. Artifacts.

(See explanation of f

Fig. 383. Artifacts.

1. Design formation of erythrocytes due to presence of fat on slide.
2. Partial hemolysis of erythrocytes due to moisture on slide.
3. Foreign material (fibers) on slide, stained blue.
4. Crystalline appearance of centers of erythrocytes due to improper fixation of slide or to presence of moisture in cells when cedar oil is added.
5. Precipitate on center of an erythrocyte.
6. Crenation of erythrocytes due to pressure when making the spread.
7. Carbon particles.
8. Precipitated stain.
9. Blood platelet on an erythrocyte.
10. Erythrocytes stained bright orange, due to acid water used in staining.
11. Erythrocytes stained pale, due to overwashing the slide or using too alkaline a water in staining.

12-17. Effect of acid water on staining of eosinophil, lymphocyte, blood platelets, monocyte, neutrophil, and another lymphocyte.

18-22. Alkaline water staining of neutrophil, 2 lymphocytes, a monocyte, and an eosinophil.

23. Alkaline staining of erythrocytes.
24. Rouleaux formation of erythrocytes, showing false staining reaction, due either to alkaline water in staining process or to improper penetration of dye into thick spread.
25. Group of blood platelets, alkaline stained.
26. Neutrophils and an eosinophil enmeshed in thick portion of spread. Erythrocytes partially dissolved, probably due to alkaline water.
27. Endothelial elements squeezed out of tissues.
28. Fibrin, due to partially clotted blood.
29. Crushed neutrophils.
30. Crushed lymphocyte.
31. Crushed eosinophil.
32. Accumulation of leukocytes on edge of a film, due to improper spreading.

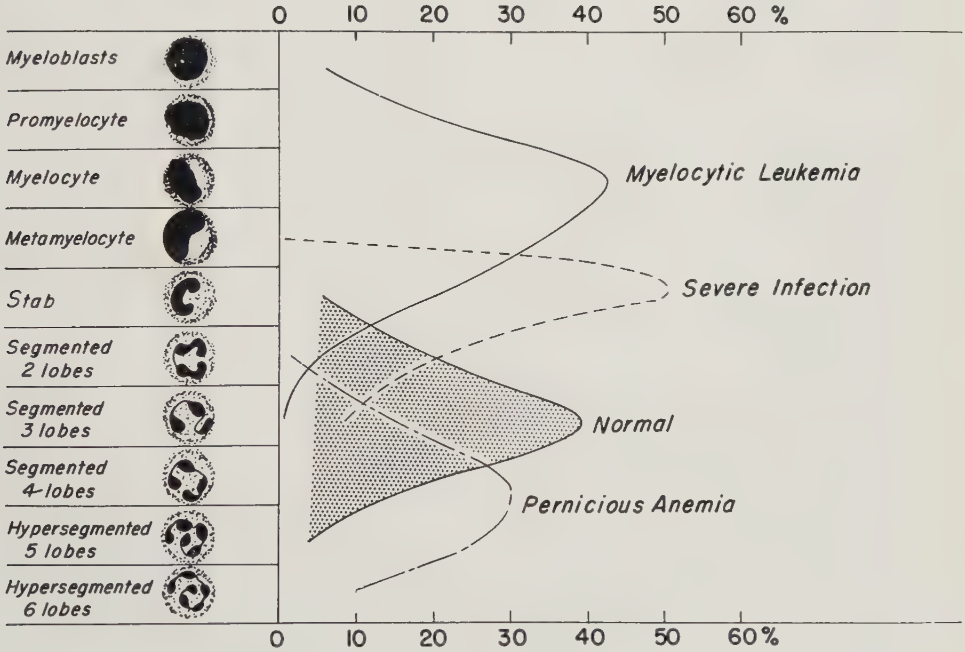

Fig. 384. Distribution of leukocytes.

basophilic. The nucleus is deep purple and contains a structure that resembles the spokes of a wheel. If the cell is overstained, the entire nucleus stains a uniform deep purple. There are no granules. Giant normoblasts are sometimes found. They are as large as megaloblasts, with small nuclei that characterize the normoblasts. Do not confuse normoblasts and lymphocytes. The polychromatic normoblast has an opaque blue or gray cytoplasm. The lymphocyte has deep blue grainy cytoplasm, with an unstained perinuclear zone usually visible. Nuclei of normoblasts are much darker than nuclei of lymphocytes, and except under conditions of degeneration they are always spherical. The round eccentric nucleus may also be confused with the nucleus of a plasma cell, but the latter has fewer chromatin spokes. The cytoplasm is basophilic and more abundant in the basophilic normoblast and orange tinged, with hemoglobin patches, in the polychromatic normoblast, and orange-red in the orthochromatic normoblast, whereas in the later phase the cell loses its nucleus, which is pyknotic and shrunken, and is expelled.

Normocytes: See discussion of erythrocytes.

Nuclear spheres: Nuclear spheres of erythrocytes are distinctly rounded droplike liquid remnants of nuclei of all sizes. They stain very dark purple. They are pathologic.

Ovalocytes: See discussion of elliptocytes.

Pernicious maculation (Mauer dots): Pernicious maculation of the erythrocytes occurs as a coarse dark violet maculation. This is seen in severe forms of falciparum malaria.

Planocytes (flat cells): See discussion of target cells.

Platycytes: See discussion of target cells.

Poikilocytes: Poikilocytes are lobulated distorted pear-shaped, oak leaf-shaped, or thorn apple-shaped degenerative forms of erythrocytes. They are found in the blood vessels following severe direct or indirect injury. They represent degenerative malformations, perhaps partially increased vulnerability. They may be artificially produced by careless technic in making the blood film.

Promegaloblasts: In size these cells are 19-27μ. The nucleus has a delicate chromatin network. There are 1-2 nucleoli present, and they are somewhat indistinct. The abundant cytoplasm is basophilic.

Polychromatic erythrocytes (basophilic stippling): Polychromatic erythrocytes are red blood cells which do not stain uniformly orange but show bluish coloration. They may or may not show stippling. Those which show stippling are called **punctated** or **stippled** erythrocytes. The term **basophilic punctation** is used to describe basophilic stippled erythrocytes. Stippling appears as fine or coarse dots stained blue or purple scattered either around the edge or throughout the entire red cell. A blood picture in which the number of polychromatic erythrocytes is increased is described as polychromasia, diffuse basophilia, or a polychromatic blood picture. One or 2 polychromatic erythrocytes may be found to every 500 normal orthochromatic cells, normally.

With the discovery of the reticulocyte in supravitally stained preparations, the question arose as to whether or not abnormal staining of this type was a sign of youth or a sign of degeneration. Hawes[196] produced evidence that the polychromatic cell of a Leishman-stained film was related to the reticulocyte of a vitally stained preparation and suggested that the stippled cell was merely a variant of the polychrome. Key,[197, 198] in 1924, confirmed the work of Hawes in relation to the polychromatic cell and pointed out that the supravital method of staining is more sensitive in detecting reticular material than is the Leishman stain for polychromasia. There is considerable doubt as to whether the stippled cell is in any way related to the reticulocyte. Piney[199] suggested that stippling may be nothing more than nuclear remnants. Samson Wright[200] in 1931, although believing that stippling is of cytoplasmic origin, did not connect it with the polychromatic cell. Maximow and Bloom[201] state: "It is possible that the stippled condition is a manifestation of immaturity much the same as polychromasia."* In this connection, Britton[202] carried out an interesting series of experiments to confirm the relation of the polychromatic cell to the reticulocyte, to establish the meaning of the phenomenon of stippling, and to determine the relation between the stippled cell and the reticulocyte and polychromatic cell. His experiments were designed to provide accurate observations on the numbers of reticulocytes, polychromatic cells, and stippled cells produced by (1) phenylhydrazine, which results in abundant polychromasia, (2) colloidal lead, which results in abundant stippling, and (3) the superposition of colloidal lead poisoning upon previously produced phenylhydrazine poisoning.

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*From Maximow, A. A., and Bloom, W.: Textbook of histology, Philadelphia, 1930, W. B. Saunders Co.

Work was done on healthy adult rabbits which were injected intravenously with 0.44% colloidal lead and with 4% aqueous solution of phenylhydrazine hydrochloride rendered neutral to phenolphthalein with sodium hydroxide. Reticulocyte counts were made with supravital staining, and counts of polychromasia and stippling were made by determining the number of these cells in proportion to red cells which were visible in an ordinary dried Leishman-stained blood film. Large doses of the drug were used in order to produce an acute poisoning. It has been shown that repeated small doses are almost without action, and that the chronic lead poisoning of human beings is almost impossible to produce in the rabbit.

This experimental work showed that reticulocytes produced by poisoning either with phenylhydrazine or with lead are, so far as can be seen from ordinary staining, identical to normal reticulocytes. The number of polychromatic and stippled cells is paralleled by the number of reticulocytes. It seemed, according to this investigator's results, that the polychromatic material, stippling, and reticulum are the same. He obtained sufficient evidence that polychromasia and stippling are both identical to reticulation. He concluded that stippling is the same chromatic substance, slightly altered by lead or other poison, as the polychromatic characteristics of red cells in general.

Basophilic stippling occurs primarily in lead poisoning but may also be seen in hemolytic anemias and following hemorrhage. The reticulum is made visible in the cytoplasm of the cells by supravital dyes. Most of these cells are young, but a few older cells may also contain the reticulum. On electron microscopic examination it can be seen that the reticulum has a definite structure. Bessis has shown that the reticulum produces indentations, grooves, and irregularities of the cell surface. Histochemically, the cell consists of ribonucleic acid and lipids.

Reticulocytes: Reticulocytes are young polychromatic erythrocytes which show a reticulum or net structure when stained with supravital methods. These nets look like fine lines throughout the cell. Two to 10 reticulocytes to every 1000 normocytes is a normal finding.

Schistocytes (erythrocytic segments): Schistocytes are irregular erythrocytic hemoglobin-containing particles which are fragments of red blood cells. They are frequently seen in severe burns, where they are associated with microcytes less than 3μ in diameter. These cells are probably the result of the direct effect of heat on the blood. Their presence may be a useful guide to the severity of the hemolysis accompanying severe burns.[203]

Schüffner punctation (dots): Schüffner punctation is a reddish violet stippling of erythrocytes occurring in tertian malaria. They are scattered over the whole erythrocyte, occasionally appearing as distinctly connected by a reticular structure.

Siderocytes: Siderocyte is a term introduced by Gruneberg[204] to refer to red blood cells which contain nonhemoglobin iron demonstrable by the Prussian blue reaction. The iron appears in the form of blue-staining granules within the red cell and has been demonstrated in normal blood, especially after splenectomy.[205, 206] (See p. 1145 for method of staining.) In the normal peripheral blood they are present in 0-3%. They are increased in some hemolytic anemias, after splenectomy, and in lead poisoning. An increase has also been reported in newborns. (See discussion of sideroachrestic hypochromic anemia.)

Kaplan et al.[207] have studied the presence of **sideroblasts** (nucleated erythrocytes giving a positive Prussian blue reaction) in bone marrow. They found them ranging from 20-90% in normal subjects. In hypochromic anemias, there is a marked reduction in sideroblasts ranging from 0.5-1.5% but usually below 1%. With intravenous iron therapy, sideroblasts return to normal values promptly. Sideroblasts are increased in thalassemia, hemochromatosis, hemosiderosis, macrocytic anemias, hemolytic anemias, and all instances of defects of hemoglobin synthesis. Björkmann[208] and Heilmeyer[208a] described a refractory sideroblastic anemia in which there is a defect in the iron utilization and an increase in sideroblasts in the bone marrow.

Spherocytes: Spherocytes are rounded red blood cells with a diameter of approximately 4μ. In blood spreads, they appear circular and seem to have a much darker stain than the normal cells; this is due to an increased thickness. They occur most frequently in congenital hemolytic icterus but may be present in occasional cases of acquired hemolytic anemia. Electron microscopic studies show thickening of the periphery which consists mainly of so-called residual hemoglobin. Chemical studies show a diminution in the lipid contents of spherocytes and suggest the possibility of an antigen-antibody reaction interfering with normal lipid synthesis.[209] The antigen-antibody theory does not apply to the congenital hemolytic anemia in which the glucose metabolism within the cell has been found to be normal, possibly due to a defect in enzymes (enolase) that control the phosphorylation of certain phosphate esters.[210]

Spherocytes are rapidly destroyed in the spleen because stagnation in the sinusoids deprives the red cells of energy-giving glucose, and because of their enzyme deficiency they are more susceptible to glucose deprivation.[211] Apart from the previously mentioned hereditary and acquired hemolytic anemias, spherocytes are also found in A-B-O hemolytic disease of the newborn, transfusion reactions, and hemolytic anemias of burns. In A-B-O erythroblastosis the spherocytes present a valuable clue as to the diagnosis since in erythroblastosis due to Rh incompatibility they are absent. Rh erythroblastosis is usually characterized by macrocytosis.

Target cells: The term target cell was used by Barrett[212] in 1938. These peculiar erythrocytes were described in a condition called target cell anemia. This type of red cell, although described by Barrett, was really first alluded to as a special type of erythrocyte by Haden and Evans in 1937.[213] They called them dimpled corpuscles. Haden and Evans believed they were present only in sickle cell anemia, but Barett maintained that they were to be found in some cases of obstructive jaundice and severe hepatitis, in some cases of hypochromic anemia, and following splenectomy. In addition to this, Dameshek[214] described a case of target cell anemia which corresponded to what he termed an "anerythroblastic type of Cooley's erythroblastic anemia."

The description of this cell, according to Dameshek, is about as follows. The cell is abnormally thin, tends to buckle, and in stained preparations presents a bull's-eye appearance. In contradistinction to the thick spherocyte, the target cells are unusually resistant to hypotonic solutions of sodium chloride. When these cells are present in any significant number (over 4%), they result in a lengthening of the fragility span, with complete hemolysis occurring between 0.16 and 0.02%, depending upon the number of these cells present. In the cases described, hemolysis was incomplete at a concentration of sodium chloride of 0.04%. He found that a high concentration of target cells was demonstrated by examination of the centrifuged red cells from concentrations of sodium chloride below 0.3%, following resuspension in the patient's own (heparinized) plasma. When the red cells were suspended in varying concentrations of hypotonic solutions of sodium chloride and the hematocrits were determined, they were found in a concentration of 0.30, 45% hematocrit, with mean corpuscular volume of 144. The target cell is the antithesis of the spherocyte.

Bohrod[215] believes that the target cell is a newly formed cell produced by the bone marrow in response to blood loss regardless of the cause. In support of this argument, he states that the appearance of target cells in the stained blood film, far from being an unusual event, is very common. Such cells can be found in small numbers, up to 1 or 2%, early in the majority of cases of anemia. Rare ones may even sometimes be seen in apparently normal spreads. In more chronic anemias the number is variable.

Target cells are of normal diameter. In the stained spread they are made up of a rim of hemoglobin-containing area, with 2 areas being separated by a zone which is clear and unstained. Barrett believed that this appearance was due to a central knoblike projection in a cup or bowl-shaped cell and showed that a body of such shape would look like a target when viewed on the broad surface. That this is actually the shape of the cell can be seen by oblique illumination of dry films or in bas-relief photographs. No target cells are seen in wet preparations. Instead, a number of cells, equivalent to the proportion which appear as target cells in the stained film, appear as shallow or more uncommonly deep bowls or cups. When blood is diluted with hypotonic saline solutions of varying concentrations, the proportion of bowl-shaped cells or of target cells in dry films increases as the salt concentration decreases. This is due to the hemolysis of the normal appearing cells and the preservation of the target cells.

Teardrop cells: These are pear-shaped cells seen in severe anemias and are suggestive of myelofibrosis.

LEUKOCYTES (SCHILLING CLASSIFICATION)

In order to interpret differential counts of the leukocytes, it is necessary to know certain facts regarding the classification of white blood cells. White blood cells are divided into granulocytes, monocytes, and lymphocytes.

Granulocytes

Granulocytes are divided as follows:

1. **Basophilic** leukocytes (or mast cells), which are present in the circulating blood in the proportion of 0.5%. They are the cells which have an affinity for the basic dyes of the Giemsa and Wright stain.
2. **Eosinophils** (or **acidophils**) are present normally in the circulating blood and comprise 2-4% of the leukocytes. They have an affinity for acids; consequently they take the eosin stain.
3. **Neutrophils** comprise about 60-70% of all leukocytes. They require a neutral dye and consequently stain a pale pinkish lavendar or bluish lavendar.

The reddish purple granules of the basophilic cells are very irregular both in size and arrangement. The eosinophilic granules are large and are very regularly distributed throughout the entire cytoplasm of the cells so that they look very much like small pale orange balls of uniform size placed as close together as possible. The granules of the neutrophilic cells are very fine and scattered unevenly throughout the cells. They stain reddish purple.

According to Schilling's classification, there are 4 divisions of neutrophilic cells: myelocytes, juvenile cells, stab cells (rod nuclear, staff form, and other terms), and segmented forms. The myelocyte is the youngest form; the segmented form is the most mature. The myelocyte has a round to oval to kidney-shaped nucleus, with large well-filled structures and a distinct trabeculated form. Myelocytes are not found normally in the circulating blood.

Juvenile neutrophilic leukocytes have a sausage-shaped nucleus. They are identical to Pappenheim's metamyelocytes. They are found only occasionally in the circulating blood in health, in the percentage of 0-1. The stab or rod nuclear cell is found normally in health in the percentage of 3-5. They have an unbroken or rod nucleus which is capable of bending or twisting so that it is seen in many different shapes. The segmented neutrophil is found normally in the percentage of 58-66 in the circulating blood. Their nuclei have 2, 3, or more distinct segments connected by bridges. The juvenile and stab cells and segmented neutrophils were all formerly referred to as polymorphonuclear neutrophilic cells.

Classification of neutrophilic leukocytes

Arneth and Cooke as well as Schilling noted that in response to an infection young forms of neutrophils appear in the peripheral blood. Their method of recording and interpreting this phenomenon differs.

The youngest neutrophil has a C-shaped nucleus with no indentation or only a very slight one, indicating beginning lobulation in the center. As this cell matures, the nucleus develops 2-4 or even more lobes.

Arneth produced a complicated classification of neutrophils which was simplified by Cooke. Cooke divided the polymorphonuclear cells into 4 groups.

Group I: unlobed nucleus or 2-lobed nucleus, the lobes being joined by a thick bridge of chromatin; normal proportion about 10% of all polymorphonuclear cells
Group II: nucleus contains 2 lobes joined by a thin filament; proportion about 25% of all polymorphonuclear cells
Group III: 3 lobes and 2 filaments; about 47% of all polymorphonuclear cells
Group IV: 4 lobes and 3 filaments; about 16% frequency
Group V: 5 or more lobes and 4 or more filaments; 2% frequency.

In infections, toxemias, and after hemorrhage, cells of Group 1 and Group 2 increase in number, producing a shift to the left which is in proportion to the degree of toxemia and is independent of the level of the total white cell count. A shift to the right is seen in pernicious anemia and in some other types of megaloblastic anemias.

The Schilling hemogram groups all leukocytes into 3 groups—polymorphonuclears, lymphocytes, and monocytes. It divides the neutrophils into 4 groups according to age—myelocytes, juvenile cells, stab (band) cells, and segmented forms.

In the Schilling count the segmented group, which represents the oldest forms, is not further subdivided.

According to Schilling an infection produces a maturation arrest of neutrophils at the juvenile level. These cells, being unable to develop further, become stab cells and remain at this level. An infection may produce 2 reactions, a regenerative reaction and a degenerative reaction. The regenerative reaction produces myeloid stimulation of the bone marrow, with release of many young forms such as myelocytes, juvenile cells, etc., into the peripheral circulation. The degenerative reaction is an expression of the toxemia of infection and produces depression of the leukocytic marrow elements so that a decreased number of neutrophils is released into the peripheral blood stream, producing leukopenia. Most of the cells released are toxic stab cells which are unable to mature and to segment further. The proportion of normal cells to toxic granulated cells is called the **degenerative index.** In most infections both reactions interplay. According to Schilling only in an infection do the juvenile and stab cells increase over the lymphocytes and monocytes. As the infection subsides, juvenile and stab cells diminish, and lymphocytes and monocytes show a relative increase. In the healing phase, juvenile and stab cells disappear, and eosinophils increase.

Monocytes

Monocytes are more than twice the size of erythrocytes. They are normally present in the percentage of 4 to 8. It is very important to differentiate these cells from lymphocytes. (See p. 1178 for complete descriptions of all the cells.)

Lymphocytes

Lymphocytes are present in the percentage of 21-30 in normal blood. They vary in size from a cell smaller than an erythrocyte to one of the diameter of neutrophilic leukocytes. (See p. 1179 for a complete description.)

Description of leukocytes stained with Wright-Giemsa stain

Myelocytic or bone marrow cells

Myeloblasts: Myeloblasts are large bone marrow cells with a narrow to wide cytoplasm and a large nucleus. The cytoplasm is usually pale or deep blue with a thready, cloudy design. The nucleus is large, reticulated, and round with 1-6 definite nucleoli. There are no granules. Frequently the cells show vacuolization. They are oxydase-positive. They are never found in the circulating blood in health. **Micromyeloblasts** are very small myeloblasts seen only in leukemia. They resemble small lymphocytic cells but have definite nucleoli and a very deeply staining blue cytoplasm. The cytoplasm frequently shows projections thought by some authorities to be pseudopodia.

Myeloblasts may contain **Auer bodies,** which are red crystalline cytoplasmic inclusions always indicative of leukemia. The Auer bodies may also be found in monoblasts but are not seen in lymphoblasts. Auer bodies are periodic acid-Schiff positive, show a crystalline pattern under the electron microscope, and are peroxidase-positive and sudanophilic.[216, 217]

The more immature a blast cell is, the more difficult is its classification. It is therefore advisable at times to call the immature cells **stem cells.** Attempts at differentiation can be made on the basis of the company the cells keep, the presence of Auer bodies, the number of nucleoli, peroxidase staining, supravital staining, and, last, fluorescent microscopy. Stained with acridine orange, the myeloblasts have an orange tinge, whereas the lymphocytic cells are greenish.

Promyelocytes or premyelocytes: Promyelocytes are large cells. The cytoplasm is usually pale blue with fine red progranules. The granules may fill the entire cell or may be scattered around the edge of the cytoplasm. If the cell is in the center of the spread, the granules at times stain blue.* The nucleus is large and round and usually shows 1 or more nucleoli. The cell is identified by the fine red progranulation and the definite nucleoli. In leukemia, promyelocytes may be very small cells, closely resembling monocytes. They may be differentiated by the oxydase stain; these promyelocytes are oxydase-positive. They stain very much darker than monocytes and show nucleoli. The differentiation is frequently very difficult.

Neutrophilic myelocytes: The cytoplasm of neutrophilic myelocytes varies from very pale blue in the younger stages to pink in the older forms. With Wright stain, it is gray and at times intensely red. The nucleus varies in shape—round, kidney-shaped, or oval. It may or may not contain 1 or more nucleoli and is trabeculated. This nuclear structure aids in differentiating it from lymphocytes with azurophilic stippling (**small** dots). In leukemia the granules of the myelocytes are very delicate and difficult to stain. They are purple and pinpoint in size. They are seen usually only here and there; at times they may be entirely absent. In the blood stream in severe infections myelocytes usually have a pale blue cytoplasm filled with coarse neutrophilic (purple) toxic granulation. The myelocyte of leukemia has a fairly even periphery, whereas that of infections is fragile and consequently may be irregular.

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*Observation of Schilling.

Text continued on p. 1177.

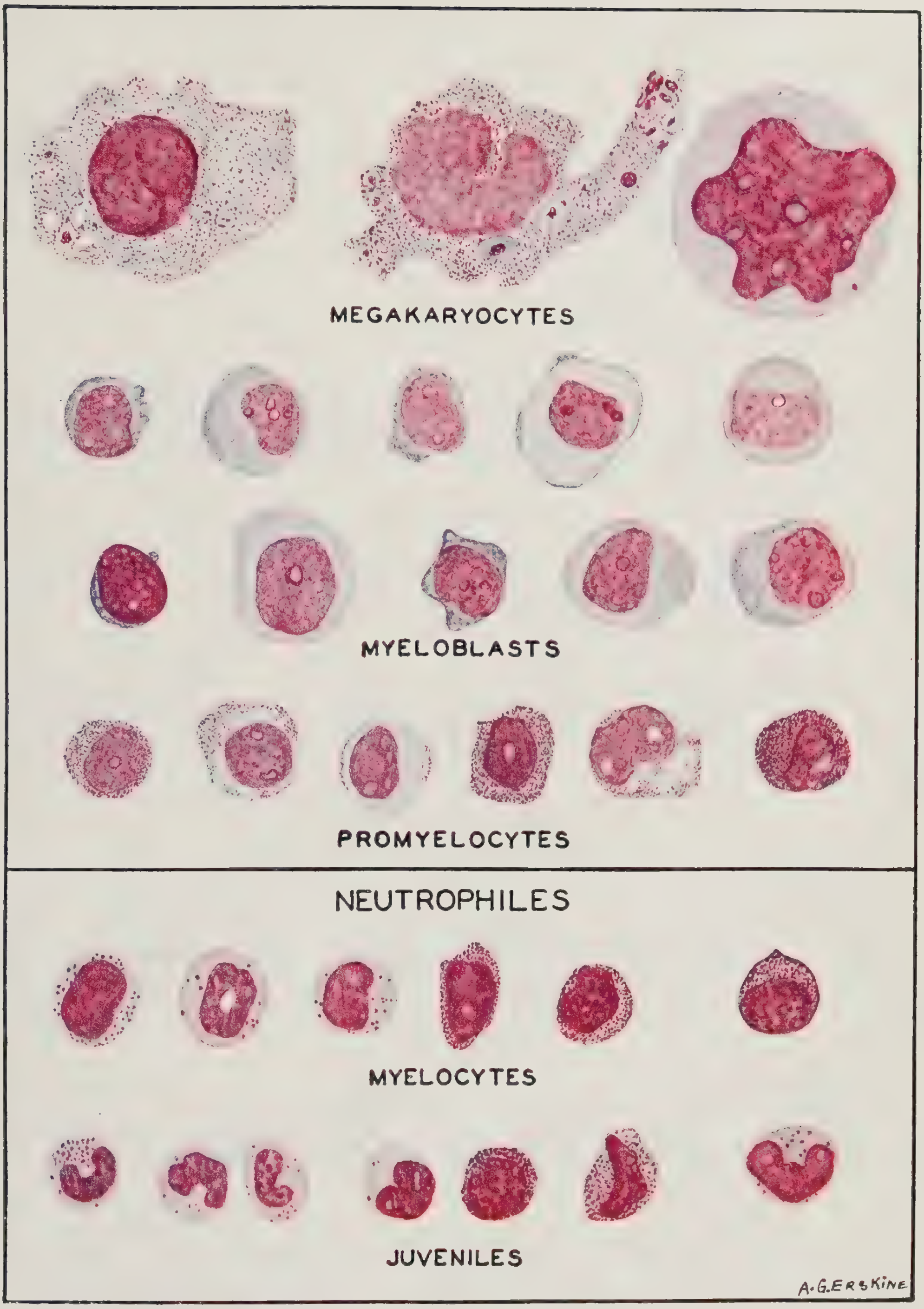
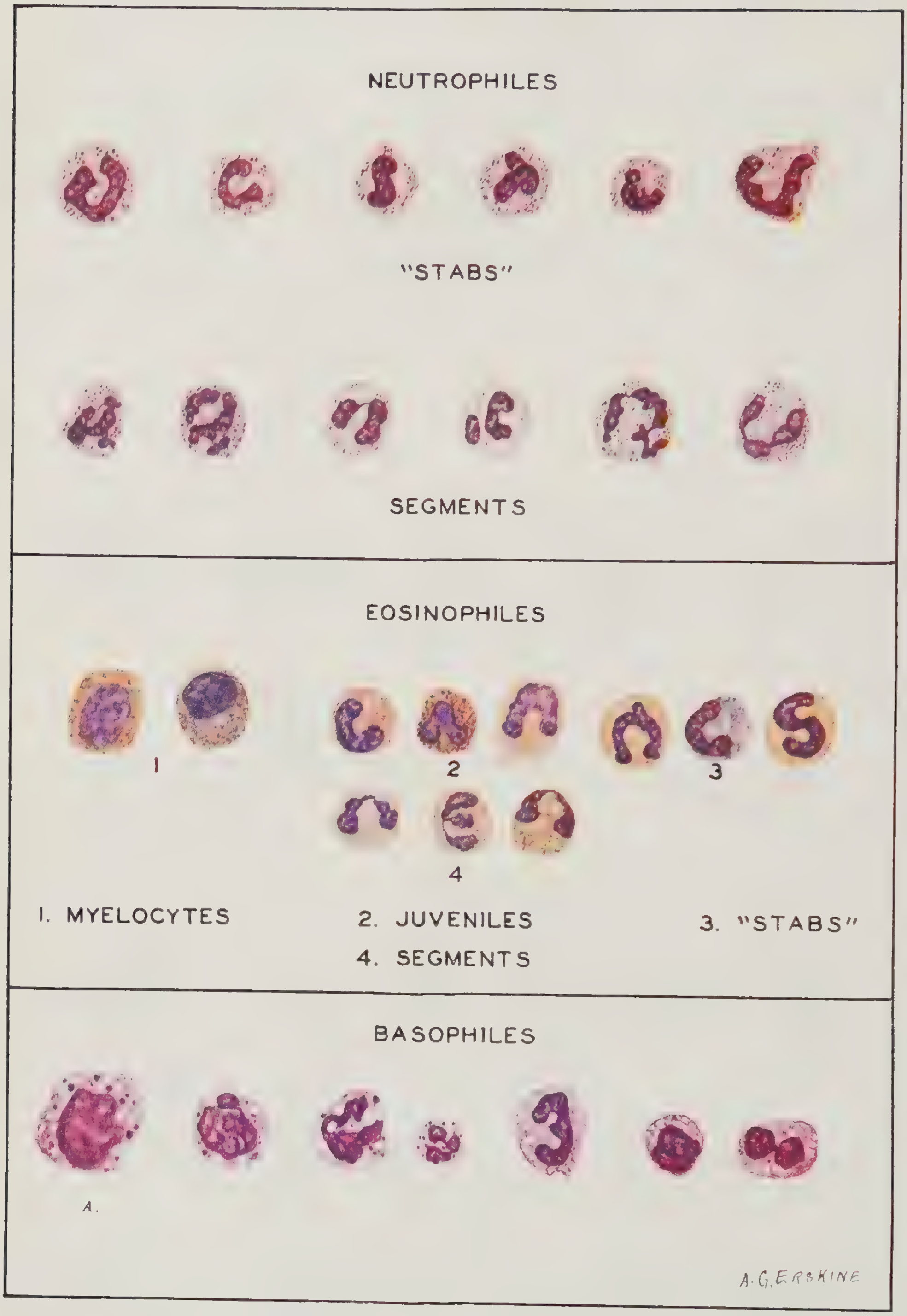

MEGAKARYOCYTES
MYELOBLASTS
PROMYELOCYTES
NEUTROPHILES
MYELOCYTES
JUVENILES
A.G.ERSKINE

Fig. 385. Leukocytes. (Giemsa stain; ×950.)


NEUTROPHILES
"STABS"
SEGMENTS
EOSINOPHILES
1
2
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4
1. MYELOCYTES
2. JUVENILES
3. "STABS"
4. SEGMENTS
BASOPHILES
A.
A.G.ERSKINE

Fig. 386. Leukocytes. **A,** Basophilic myelocyte. (Giemsa stain; ×950.)

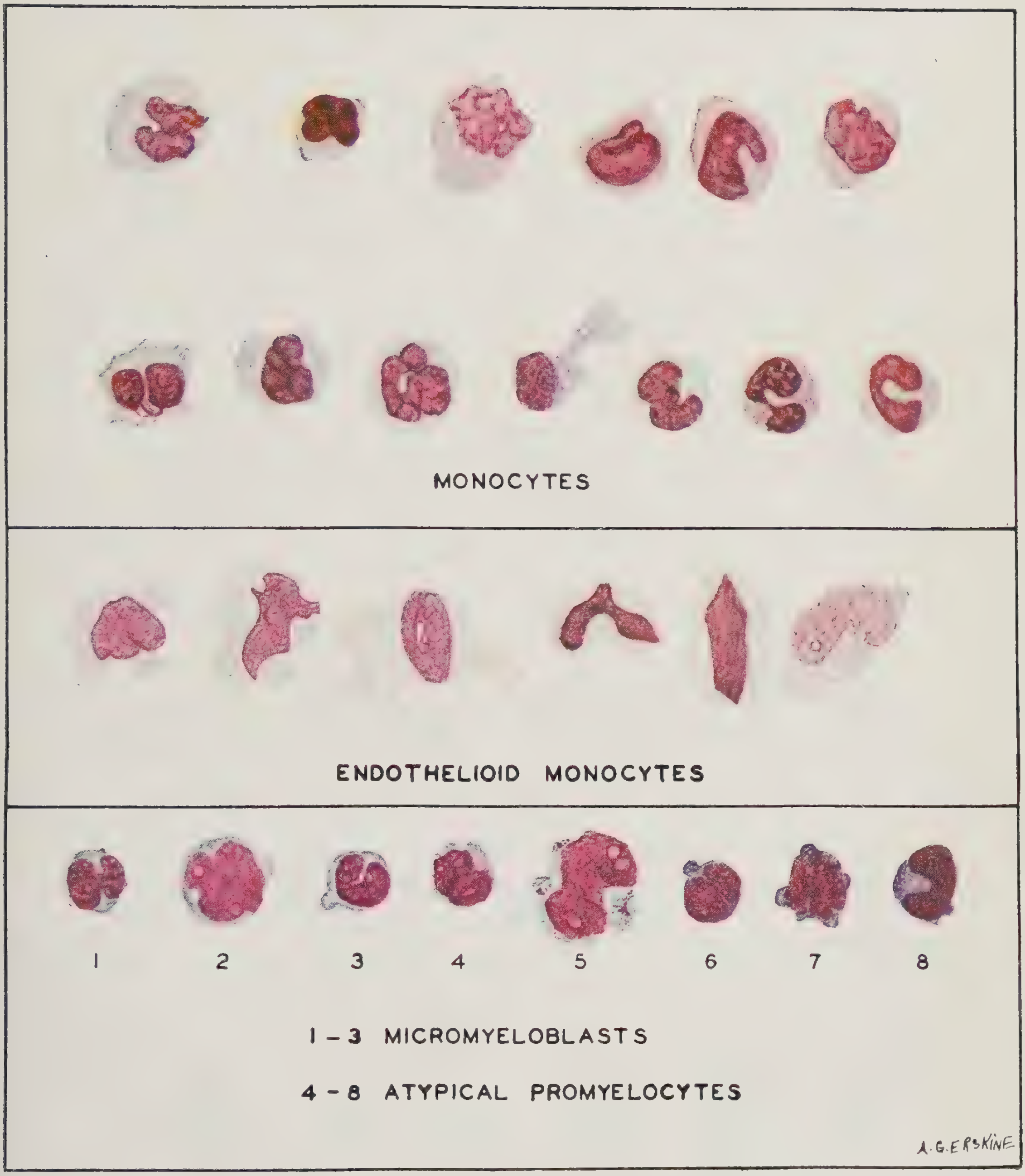
MONOCYTES
ENDOTHELIOID MONOCYTES
1
2
3
4
5
6
7
8
1 – 3 MICROMYELOBLASTS
4 – 8 ATYPICAL PROMYELOCYTES
A. G. ERSKINE

Fig. 387. Leukocytes. (Giemsa stain; ×950.)

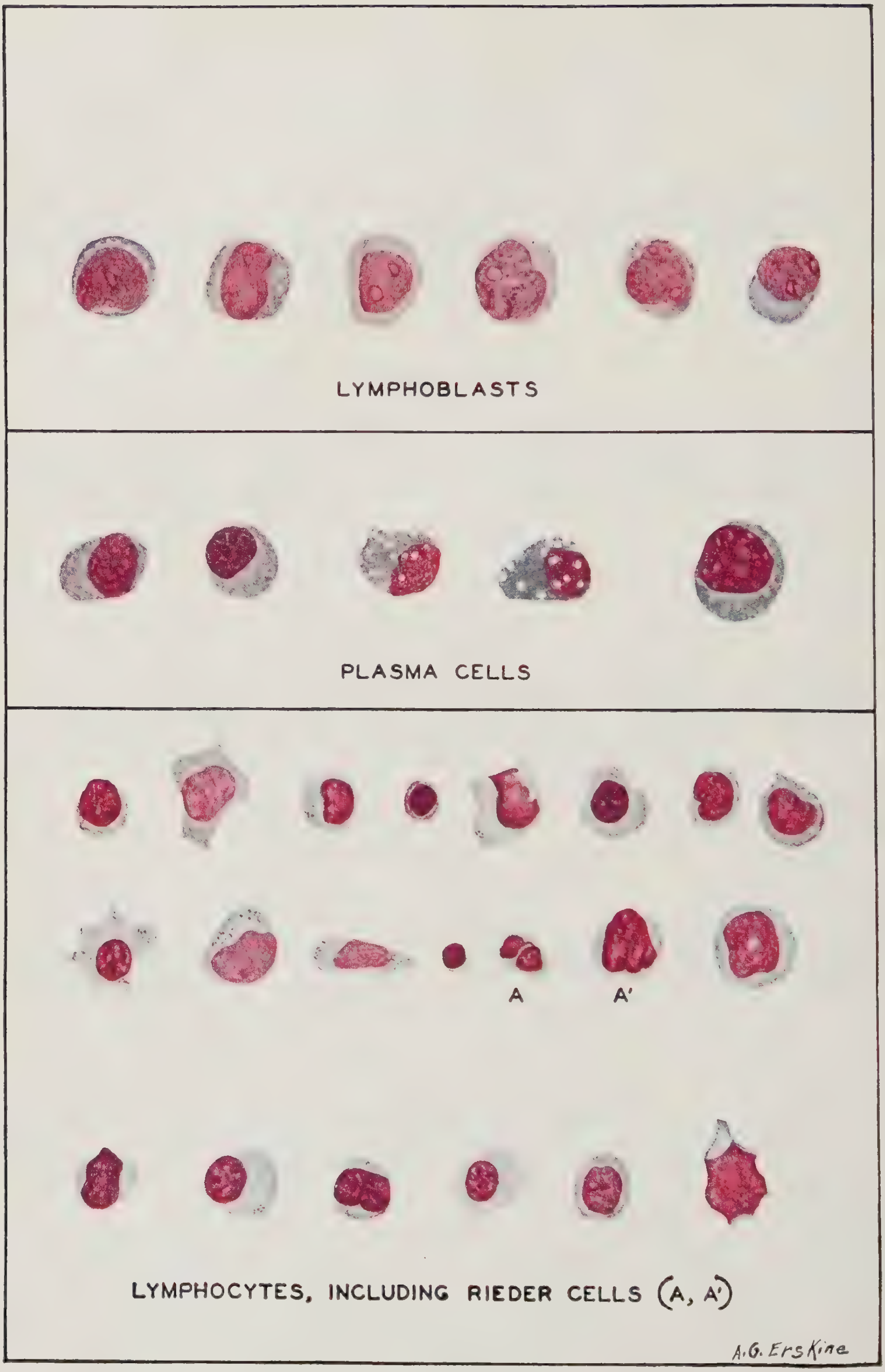
LYMPHOBLASTS
PLASMA CELLS
A
A'
LYMPHOCYTES, INCLUDING RIEDER CELLS (A, A')
A.G. Erskine

Fig. 388. Leukocytes. (Giemsa stain; ×950.)

Juvenile neutrophils or metamyelocytes: Juvenile neutrophils are found normally in the circulating blood in the percentage of 0-1/100 leukocytes. They are slightly larger than the mature neutrophils. The cytoplasm varies from a bluish pink to a definite pink or gray, particularly when stained with Wright stain alone. It is much wider than that of the myelocytes. The nucleus is sausage-shaped to almost bean-shaped. It does not stain intensely and shows little or no chromatin structure although it is definitely divided into fields. The granules are sometimes definite and sometimes very fine. When they are coarse, they are strikingly responsive to stain; when fine, they stain with difficulty. The same variations in staining and structure may be noted in leukemia and infections as are seen in the myelocytes. They are differentiated from stab cells by the pale nucleus without chromatin, the nucleoli, and the width of the nucleus. Stab cells are older and consequently stain more deeply, have no nucleoli, show dense chromatin structure, and have a fairly narrow nucleus. When a cell has developed beyond the juvenile stage but has not quite become a stab (borderline cell), Schilling suggests that it be called a stab.

Giant metamyelocytes are consistently found in pernicious anemia.

Stab, band, or rod nuclear neutrophilic cells: The term stab, originated by Schilling, is a German word which refers to the ability of the rubberlike rod-shaped nucleus to become bent or twisted. Stab cells are neutrophilic cells and are found in the normal circulating blood in the percentage of 3-5. The cytoplasm is pink or lavender, often gray with Wright stain. The nucleus is a flexible rod, which may be bent in making the film, so that it takes the form of the letters S, T, U, V, or W. It does not show segmentation although degeneration forms in which the nucleus is vacuolated or fringed may occur. Any cell which shows even slight segmentation is not considered a stab cell. The nucleus contains a dark chromatin structure which must not be mistaken for nucleoli. It stains more intensely than the nucleus of the juvenile cell and is slightly narrower. It does not contain nucleoli. The granules are usually distinct, very fine, purple-staining stippling and are distributed less regularly than the granules of eosinophils but more regularly than those of basophils. Under conditions of severe infections, these are very coarse and clumped. (See discussion of toxic granules, p. 1177.) When the cell is very definitely in the borderline stage between a stab and a segmented form, i.e., if it has developed farther than the stab stage, Schilling suggests that it be called a segmented cell.

It is important to remember that there are **degenerative** forms of stab cells. The degenerative forms show small band forms of nuclei—irregular, hyperchromatic, pyknotic, and structureless. They are easily broken up in making the preparation. There is a tendency toward vacuolization and diminished resistance. They are a result of a toxic element or infection. The Pelger-Huët anomaly is discussed on p. 1178.

Segmented neutrophils: Segmented neutrophils are found normally in the circulating blood in the proportion of 58-66/100 leukocytes. The diameter of the cell is about one and one-half that of an erythrocyte. The cytoplasm is usually a clear pale pink or lavender. The nucleus is usually divided into 2-5 irregular segments which are connected in a chain by fine threads or bridges. They show deep-staining chromatin structure and no nucleoli. The granules are fine, light-refracting, pale reddish violet, except in severe infections, in which they are coarse and massed in irregular clumps. These so-called **toxic granules** are also seen in the presence of malignant tumors and in splenic diseases. They may represent persistance of a myelocytic type of granulation. **Alder's anomaly**[218] consists of basophilic granulation of the neutrophils and eosinophils and is associated with gargoylism. Severely toxic segmented neutrophils may contain 2-4 pyknotic nuclear masses which are not connected and may show nuclear cytoplasmic dissociation.

Abnormalities of polymorphonuclear leukocytes

Giant hypersegmented polymorphonuclear leukocytes are usually found in pernicious anemia. The administration of colchicine, a drug that blocks mitosis, also produces giant neutrophils. The production of **giant neutrophils,** as well as of hypersegmentation of the nuclei, has been described as 2 separate types of inherited anomalies.[219]

The cytoplasm of neutrophils may show pathologic changes apart from the previously mentioned toxic granulation and Alder's anomaly. Döhle described basophilic patches in toxic polymorphonuclear cells. They are similar to the inclusions described by Hegglin,[220] but Hegglin inclusions are also found in monocytes and in lymphocytes and are associated with abnormal giant platelets. **Döhle bodies** are also somewhat similar to the irregular inclusions found in **Chediak's[221] syndrome,** which is characterized by semialbinism and enlargement of the spleen, liver, and lymph nodes.

In the **Pelger-Huët's anomaly** the nuclei of the polymorphonuclear leukocytes are not segmented but are similar to the nuclei of stab and juvenile cells. This disorder is hereditary and consists of an inability of nuclei to segment so that an apparent shift to the left is produced.[222]

This anomaly is exactly the opposite of the inherited anomaly of hypersegmentation described by Undritz.[223]

Eosinophils: Eosinophils are found normally in the circulating blood in the proportion of 2-4/100 leukocytes. The clear white or pale blue cytoplasm is composed of many highly light-refracting granules which stain a faint orange to a decided orange with the Giemsa stain. With the Wright stain, they are more intensely stained. They are usually situated so close together that they give the appearance to a casual observer of an almost uniformly orange-stained cytoplasm. The granules may be rendered more pronounced by partially closing the iris diaphragm. In young eosinophilic cells, or in mature cells if alkaline water is used in staining, there are frequently found deep blue granules. Sometimes the entire cytoplasm stains a uniform gray color, but the identification of the cell can still be made by the regularity of the granules and the shape of the nuclei. Bright crystallike granules may also be seen. This is an artificial picture due to the highly light-refracting characteristic of the granules.

Eosinophils are differentiated as eosinophilic myelocytes (if they have round or oval nuclei), eosinophilic juvenile cells (if the nuclei are like metamyelocytes), eosinophilic stab cells (with typical stab nucleus), or segmented eosinophils. It is customary not to classify the eosinophils except as myelocytes and segmented eosinophils (simply eosinophils) except when eosinophilic myelocytes are found.

The nucleus of the mature eosinophil usually has 2 segments, double pear-shaped. There are seldom more than 3 or 4 segments although it is possible to find them. Eosinophils retain their characteristics in the thick drop. To intensify the orange color of the granules, make the staining water slightly acid.

Eosinophils are responsible for the production of Charcot-Leyden crystals and of plasminogen. They are present in the stool, blood, sputum, and nasal secretions of patients suffering from allergic diseases.

Basophils or mast cells: Basophils may also be divided into basophilic myelocytes and mature cells. The mature forms are found normally in the circulating blood in the proportion of 0-1/100 leukocytes. The cytoplasm is usually very narrow and stains deep reddish purple with the Giemsa stain. With the Wright stain the cytoplasm is often blue. It is composed almost entirely of irregular basophilic fragments which usually stain intensely. These fragments are very highly soluble in water and therefore drop out of the cytoplasm during the staining process, leaving the cell appearing highly vacuolated. If the granules do not dissolve, they stain intensely reddish purple and are unevenly distributed throughout the cytoplasm. With Wright stain the granules stain a dark blue-purple. The nucleus is bizarre and lobulated and occupies most of the cell. It stains a deep reddish purple with the Giemsa stain and an intense bluish purple with the Wright stain.[224]

An increase in basophils can be seen in myelogenous leukemia but is also found in myeloproliferative disorders. The blood basophil may be only functionally related to the tissue mast cell. Both have the same function of storing and producing heparin, histamine, and serotonin. The tissue mast cell, though somewhat similar in appearance to the blood basophil, is a connective tissue cell and is frequently found in bone marrow. The tissue mast cell is larger than the blood basophil, it is oxidase negative, and the granules do not dissolve in methyl alcohol. There is a large amount of cytoplasm, and the cell is never found in the peripheral blood.

An increase of tissue mast cells has been reported in macroglobulinemia. The basophilic granulocyte should be clearly separated from the connective tissue mast cell. Mast cell proliferation, even leukemia, has been described in association with urticaria pigmentosa (mastocytosis) and also in association with adrenal cortical hyperfunction and with anaphylaxis.[225, 226]

Cells from reticuloendothelial system

Monocytes: Monocytes were formerly called large mononuclear cells and transitional cells. They are found normally in the circulating blood in the proportion of 4-8/100 leukocytes. They are usually much larger than the granulocytes but may be as small as a small lymphocyte. The cytoplasm is usually very wide, is smoky blue to pale pinkish violet or blue, and is frequently covered with a very fine pink dust. It may or may not show vacuoles. The cell shows a very irregular outline, which helps differentiate it from lymphocytes. The nucleus is fairly large and may vary from oval to bean-shaped or kidney shape or may even show lobulation. Occasionally it closely resembles the nucleus of the juvenile neutrophil. It can easily be differentiated by the color and structure of the cytoplasm. Sometimes a sausage-shaped nucleus is seen, and at times nucleoli are distinctly visible. The edges are seldom if ever smooth; the contour appears wrinkled instead of smooth or flat. Monocytes are usually granulated, the cytoplasm being filled with very fine pink dustlike granules which when stained intensely may be mistaken by the beginner for neutrophilic granules. They are, however, less distinct than neutrophilic granules and are possible of differentiation. Very large phagocytic monocytes have been called **macrophages.** Monocytes are slightly oxidase-positive.

There are ill-defined boundaries between monocytes, lymphocytes, and plasma cells. In the bone marrow of macroglobulinemia various transition forms have been described.[227] The term **virocytes** has been coined to describe cells which have characteristics of both lymphocytes and monocytes and which appear in virus diseases such as infectious mononucleosis, hepatitis, viral upper respiratory infections, roseola, etc. These cells are probably transitional forms which may correspond to the cells which make up the germinal center of lymph nodes.

Unlike the lymphocytes, the monocytes do not show a perinuclear unstained zone. Differentiation of these cells from lymphocytes is made as follows.

1. Monocytes are usually larger than lymphocytes, but the size is not a point in differentiation if all other characteristics show the cell to be distinctly monocytic.
2. The nucleus of the monocyte is wrinkled; that of the lymphocyte is smooth and solid and usually shows deeply stained chromatin material. The monocyte occasionally shows nucleoli.
3. The structure of the monocyte is reticulated and more delicate than the structure of the lymphocyte; therefore, the cell stains paler than the lymphocyte.
4. The monocytes tend to show great variations in the shapes of the nuclei.
5. The lymphocyte usually shows a clear, unstained perinuclear zone, whereas the monocyte is usually devoid of such a zone.
6. The monocyte shows a pink, powdery granulation, whereas the lymphocyte is either without granules or shows azurophilic stippling.
7. The cytoplasm of monocytes is smoky blue, gray, or lavender, whereas that of lymphocytes is a definite clear pale or deep blue.

Monoblasts: Monoblasts are immature monocytic cells and are larger than mature monocytes. They are never found normally in the circulating blood. They are irregular in shape. The nucleus is in the form of an irregular crescent or is broad and elongated. It might be bent over itself several times, giving the impression of lobulation. It has a loosely woven chromatin network and often stains paler than lymphoblasts and myeloblasts. The cytoplasm is more abundant and less basophilic than that of lymphoblasts. Azure granules may be seen; vacuolization is common. They exhibit lively ameboid movements and may be either oxidase positive or negative. The cytoplasm may contain Auer bodies. (See discussion of myeloblasts.)

Endothelial cells: Endothelial cells are long caudate cells and frequently have a conspicuous oval, eccentric, finely designed nucleus. At times nucleoli are visible. The cytoplasm is very delicate and if visible stains either very pale pink or very pale blue. There seem to be fine pink granules or dust throughout the irregular cytoplasm, although this may be absent.

Lymphocytic cells

Lymphoblasts: Lymphoblasts are never found normally in the circulating blood. The cytoplasm is usually deep blue with a threadlike cloudy design appearance. It is usually narrow and has a smooth edge. At times, however, cytoplasmic projections said to be pseudopodia are observed. The nucleus is large and reticulated and may have 1 or 2 nucleoli, occasionally 3. There are no granules. The cells are oxidase-negative, and Auer bodies are never found. Some lymphoblasts resemble reticulum cells somewhat, whereas others resemble myeloblasts and have a nucleus with a delicate chromatin pattern. This form is frequently seen in acute leukemia. Atypical forms with lobulated, indented, and folded nuclei have been described as **Rieder cells.** Similar forms also occur in lymphocytic leukemia.

Lymphocytes: Lymphocytes are found normally in the circulating blood in the proportion of 21-30/100 leukocytes. Some authorities consider 35% lymphocytes a normal finding. They have been grouped as large and small lymphocytes, the large lymphocytes comprising about 6% of all the blood leukocytes. They are increased in thyrotoxicosis. The cytoplasm varies from very pale, almost invisible, clear light blue to deep blue, always clear. The width of the cytoplasm varies from a very narrow band to a very broad, smooth cytoplasm. Occasionally no cytoplasm is visible. There is usually an unstained perinuclear zone clearly visible. The nucleus is always stained deep blue or violet. It may show a deep-staining chromatin structure although the general appearance is smooth. It may be round, oval, or indented. It may be central or eccentric. Sometimes the nucleus shows distinct lobulation or segments (**Rieder forms**). These forms are pathologic. There are no granules, although azurophilic stippling is not uncommon. There is some evidence that increased gamma globulin is related to the production of these granules.

In the discussion of monocytes, mention has been made of transitional forms which combine features of lymphocytes, plasma cells, and reticulum cells as seen in macroglobulinemia or in virus diseases such as infectious mononucleosis and hepatitis. These cells are called **virocytes** or **atypical lymphocytes.** The older term of Türck's irritation cell also refers to an atypical lymphocyte of infectious origin, with dark blue cytoplasm and an eccentric nucleus.

In lymphocytic leukemia many abnormal forms appear, such as smudged cells (basket cells), spindle-shaped cells, naked nuclei, and the previously mentioned Rieder cells. By specialized method, such as supravital staining, phase microscopy, and electron microscopy, various cytoplasmic inclusions can be seen in lymphatic leukemic cells. These inclusions are of lipid, mitochondrial, and crystalline nature.

Wiseman, in attempting to evaluate the age of lymphocytes, studied normal adult human and rabbit bloods. As a result of his research, he stated that basophilia is manifestly a criteron of youth, but the absence of it in a given cell does not necessarily mean that the cell is mature.

For a description of lymphocytes in infectious mononucleosis, see p. 1332.

Plasma cells: Plasma cells are rarely found in the circulating blood in health. The cytoplasm stains intensely blue, with a fine reticular blue structure often showing vacuoles. The nucleus is usually eccentric and is surrounded by an unstained clear perinuclear zone.

In the bone marrow, plasma cells often have 2-4 nuclei. The cytoplasm may show inclusions, such as crystals, hyaline droplets, and Russell bodies. The last are protein (or paraprotein) structures derived from mitochondria as provided by electron microscopy. The mitochondria of the plasma cell are probably responsible for the production of gamma globulin. Electron microscopy reveals a well-developed ergastoplasm.

Megakaryocytes and blood platelets

Megakaryocytes are giant bone marrow cells never seen in the blood stream in health and very rarely found in the blood stream in disease. They are seen pathologically in the spleen and in the lungs, though a few can be found in both organs under normal conditions. They are very large finely granular faintly colored cells. The cytoplasm is pale blue or lavender. The nuclei show bizarre, antlerlike deep lobulations; they contain 1, 2, or more, sometimes as many as 12, of these nuclei. They are covered with a faint azurophilic dust.

Megakaryocytes are rarely found in the circulating blood. Megakaryocytes or megakaryocytic fragments have been seen in leukoerythroblastosis, myelocytic leukemia, erythremia, and Hodgkin's disease, in cases in which leukocytosis was present due to infection, and in all forms of myeloproliferative syndrome.

Megakaryoblasts: These are cells about twice the size of myeloblasts with a blue nongranular cytoplasm and a large, somewhat irregular single nucleus, which at times is kidney-shaped, with numerous nucleoli which cannot be seen with Wright stain but require special stain. These cells do not produce platelets. In normal preparations they comprise less than 1% of all the megakaryocytes.

Promegakaryocytes: These cells are usually about the same size as the megakaryoblast, although occasionally much larger, with scant dark blue cytoplasm and a dense nonlobulated or partly lobulated nucleus with heavy chromatin. These forms frequently have plateletlike bodies at the periphery of their cytoplasm and rarely nongranular cytoplasmic processes. Normally, promegakaryocytes make up about one third of the total number of megakaryocytes. They are especially numerous in the bone marrow of infants.

Adult megakaryocytes: This cell is of variable size, but is usually very large, and contains a single large multilobed nucleus, with increasing density as it matures. The cytoplasm varies in color from blue to pink and contains a variable number of characteristic azurophilic granules grouped at first in the perinuclear zone. Typical platelets which are almost always granular are frequently found in pseudopod-like structures grouped in masses about the periphery of the cell. The distribution of these cells in the normal bone marrow is uneven and depends somewhat on the technic used. There should be 1-3 megakaryocytes per low-power field of bone marrow smear. In bone marrow sections, the number appears to be increased, but the distribution can be judged more easily.

Degenerated forms: In these cells the cytoplasm is either homogeneous and hyaline in appearance, there is marked vacuolization of the cytoplasm, or the nucleus is hyperlobulated and its cytoplasm is nongranular. Frequently naked degenerative megakaryocytic nuclei can be seen surrounded by platelets. Polykaryocytic forms of megakaryocytes cannot be easily separated from osteoclasts. Such forms are seen in pernicious anemia and in idiopathic thrombocytopenic purpura.

It must be recognized that the relationships of the various types of cells to each other may be more artificial than real since they are based on a study of what appears to be transition forms. Because of this uncertainty, the megakaryoblasts and promegakaryocytes may be designated young forms and the adult types adult forms, with a separate designation for the degenerated cells.

For further information, see the discussions of platelets and idiopathic thrombocytopenic purpura.

Nonhematologic cells found in film of peripheral blood

Three groups of cells may be found: (1) fungi (*Histoplasma capsulatum* in monocytes), (2) parasites (malaria and *Bartonella* in red blood cells), and (3) tumor cells.

The first two will be considered in the discussion of hemolytic anemia.

Tumor cells in peripheral blood[228-231]: Since carcinoma spreads by lymphatic and hematogenous routes, tumor cells may be found in the peripheral blood film. Because the finding of tumor cells in the routine peripheral film is rather unusual, methods have recently been developed to concentrate the tumor cells circulating in a given quantity of blood. These methods utilize Millipore* filters and enzymes. The presence of cancer cells in the peripheral blood may warn the physician that even after successful surgery the patient will require some other type of therapy. At the present time these concentration methods are somewhat tedious and probably somewhat inadequate because of the small size of the blood sample (5-10 ml.) and because of the difficulties in recognizing circulating cancer cells. It may well be that the Millipore filter technic in conjunction with the acridine orange fluorescent technic will give better results.

METHODS OTHER THAN WRIGHT OR WRIGHT-GIEMSA USED IN STUDY OF BLOOD MORPHOLOGY

The cell description contained in the preceding pages refers to blood cells stained with Wright, Giemsa, or Wright-Giemsa stains.

In recent years new methods have been utilized to discover the finest details of blood cell morphology, or older methods have been adapted and modified for similar purposes.

Dark-field examination

Dark-field examination is based on the fact that objects which are illuminated by oblique light which is not allowed to enter the objective will reflect and scatter the light rays. They thus appear brilliant against a dark background.

If unstained blood is examined with the dark-field microscope, the white cells are difficult to recognize, with the exception of the eosinophils and basophils which contain prominent granules. The erythrocytes are seen as pale yellow disks. For the investigation of reticulocytes and Heinz bodies, dark-field examination after supravital staining is satisfactory. It is equally applicable to the examination for basophilic stippling. Dark-field examination allows the visualization of platelets and of numerous plasma structures of doubtful significance.

Phase microscopy

Phase microscopy allows the observation of unfixed living cells without any prior treatment. Phase microscopy transforms differences in the refractive index of the various cell structures into degrees of brightness and contrast. It thus allows the observation of living mitochondria and other intracytoplasmic structures. Outstanding contributions to the subject of hematology and phase microscopy will be found in the papers of Bessis,[232] Ackermann, and Bellios.[233]

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*Millipore Filter Corp., Bedford, Mass.

Description of most important cells as they appear in phase microscopy

Neutrophils: These cells measure 12-18μ in diameter. The cell has ameboid movement. The nucleus is dense and is surrounded by cytoplasm which shows intracytoplasmic movement, specific granulation, and phagocytosing vacuoles. The myeloid precursor form shows nucleoli and numerous mitochondria.

Lymphocytes: The nucleus shows a dense chromatin pattern interrupted by a small nucleolus. The cell shows movement in which the nucleus precedes the cytoplasm. The latter is seen to contain mitochondria.

Monocytes: The nucleus has a delicate chromatin pattern and frequently contains 1 or 2 small nucleoli. The cytoplasm contains mitochondria and during movement produces flat, broad pseudopods.

Erythrocytes: If red cells contain Howell-Jolly bodies or Heinz bodies, these can easily be seen with phase microscopy. The nucleus of erythroblasts contains nucleoli, and the cytoplasm contains perinuclear mitochondria. The dense normoblastic nuclear chromatin pattern can also be seen.

Platelets: The pseudopodia and the division into hyalomere and granulomere can easily be seen when platelets come in contact with a surface. Phase microscopy is the basis for one of the best methods of platelet counting.

Fluorescent microscopy

Fluorescent microscopy allows recognition of substances within tissues by their ability to fluoresce. When these substances are exposed to invisible ultraviolet light of short wavelength, they absorb this light and are thus excited. In this excited state they emit visible rays of light. Some tissues fluoresce naturally, whereas others fluoresce only after staining with certain dyes (primary and secondary fluorescence).

Fluorescent technic is extremely sensitive and is able to detect dye concentrations in the order of $1:10^{-18}$.

In erythropoietic porphyria, fluorescent erythrocytes and erythroblasts can be seen (**fluorescytes**). Similar cells in small numbers have been reported in untreated pernicious anemia. Fixed bone marrow and blood films can be stained with acridine orange and coriphosphin O. In the erythroid series the more mature a nucleus, the more brilliantly will it be stained, whereas the cytoplasm stains more vividly the younger the cell is; i.e., the less hemoglobin it contains. Heinz bodies can be recognized under ultraviolet light in the air-dried preparation. As far as the white cell series is concerned, living leukocytes have a delicate primary fluorescence which increases as the cells die. Fluorescent microscopy, as well as phase microscopy, has been used to show the production of platelets from megakaryocytes.

Electron microscopy

Electron microscopy allows two approaches—magnification up to 200,000 times

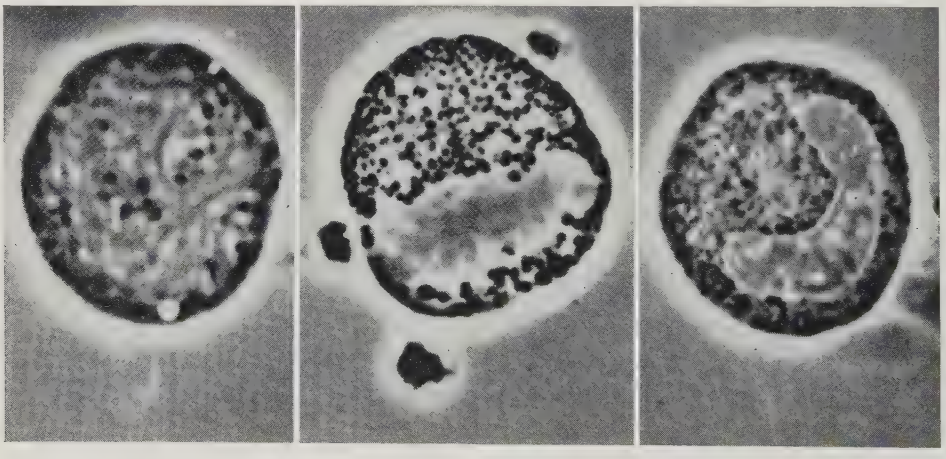


Fig. 389. Phase contrast photomicrographs of granulocytes. **A,** Neutrophil. **B,** Eosinophil. **C,** Basophil. (From Bernard, J., and Bessis, M.: Hematologie clinique, Paris, 1958, Masson & Cie.)

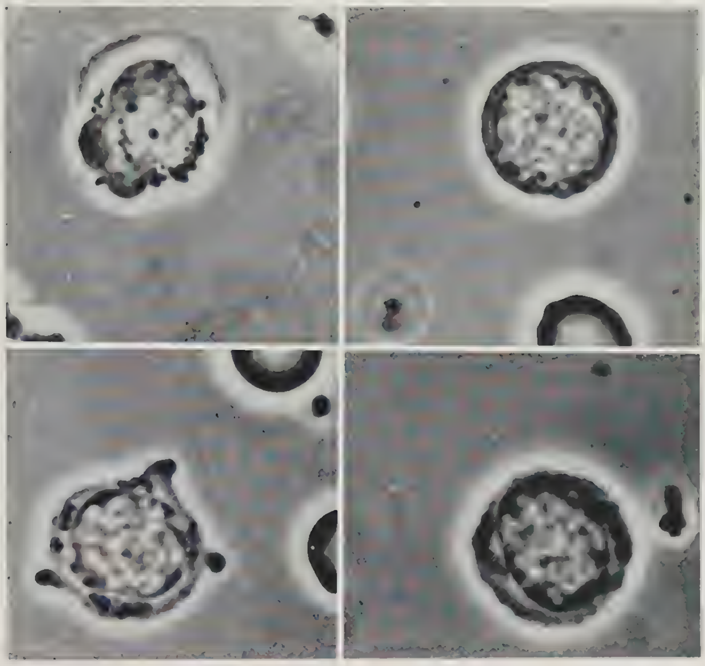


Fig. 390. Phase contrast photomicrographs of small lymphocytes. (From Bernard, J., and Bessis, M.: Hematologie clinique, Paris, 1958, Masson & Cie.)

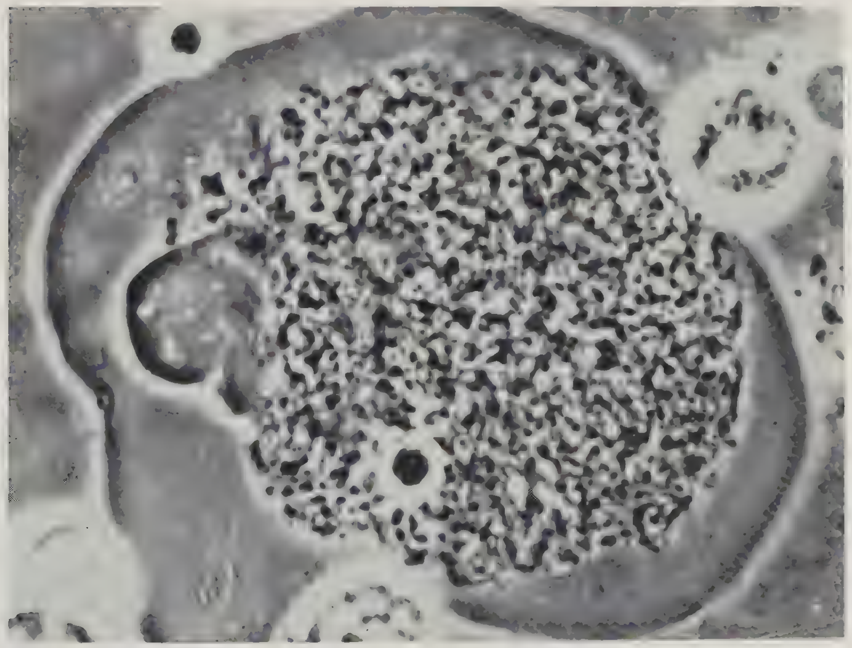


Fig. 391. Phase contrast photomicrograph of granular megakaryocyte. (From Bernard, J., and Bessis, M.: Hematologie clinique, Paris, 1958, Masson & Cie.)

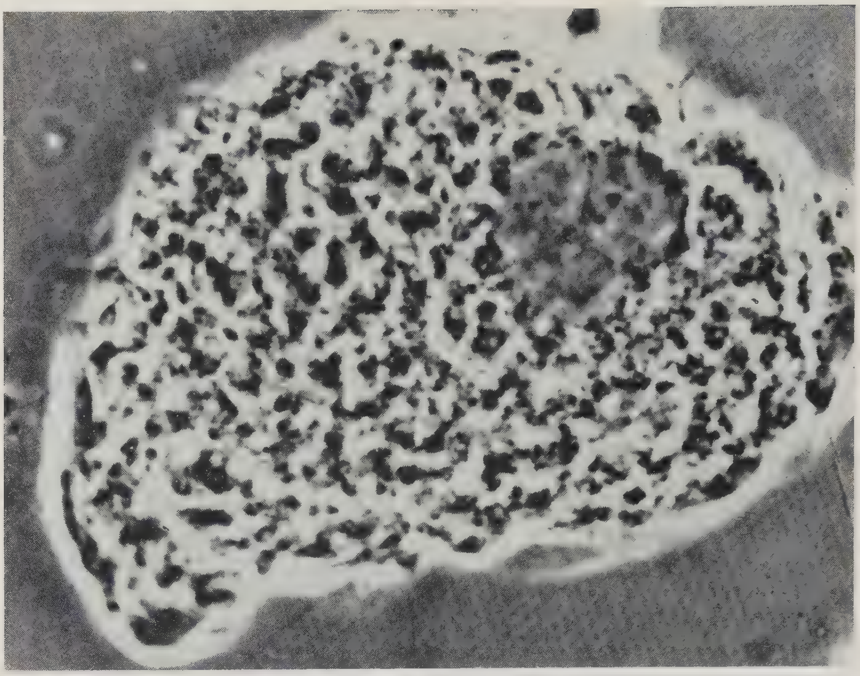


Fig. 392. Phase contrast photomicrograph of megakaryocyte showing division of cytoplasm in areas due to developing platelets. (From Bernard, J., and Bessis, M.: Hematologie clinique, Paris, 1958, Masson & Cie.)

of sections cut at 0.02μ thickness and magnification up to 1000 times of unstained cells.

Erythrocytic series: Bessis and others investigated normal and abnormal red blood cells and found that the surface of a normal red cell shows a few craterlike depressions. Under the influence of hemolytic substances, these craters increase in depth, and the surface becomes irregular. The surface of the reticulocyte is very irregular, with numerous folds, craters, and fissures. The investigation of the stroma of the red cell exposed to a hemolytic system shows movement of the residual hemoglobin from the periphery of the cell to its center. Studies of iron metabolism show beautifully the destruction of red blood cells within phagocytes, the liberation of ferritin, the transfer of the phagocytosed ferritin from the reticulum cell to the erythroblast by the process of pinocytosis, and finally the transformation of the ferritin into erythroblastic mitochondria. Later in the development of the cell, this iron is again liberated and is incorporated into the hemoglobin molecule.

Leukocytic series: The nuclear pattern depends on the chromatin contents. The nucleus is surrounded by a cell membrane and contains a darker nucleolus. Electron microscopy allows the visualization of mitochondria. Each mitochondrion is surrounded by a membrane carrying cristae on its side. The granulations of the various leukocytes vary somewhat in appearance according to their age and type. Many of the intracytoplasmic structures such as the Golgi apparatus and the centrosome have been investigated.

Under pathologic conditions changes have been seen in nuclei, in mitochondria, and in intracytoplasmic structures. The bone marrow cells have also been investigated, and most of the work on the transport and utilization of ferritin has been done on bone marrow preparations. Electron microscopic investigation of the megakaryocyte shows its cytoplasm to contain small canaliculi which surround small cytoplasmic areas so that the cytoplasm has a definite fielden pattern somewhat reminiscent of platelet formation within the cytoplasm.

Making and staining thick drop preparations

The thick drop method of examination of blood is an important procedure in searching for parasites.

Parasites of malaria, filaria, trypanosomes, etc., may be found more readily in the thick drop than in the blood film. Often these parasites may be seen in the thick drop but not

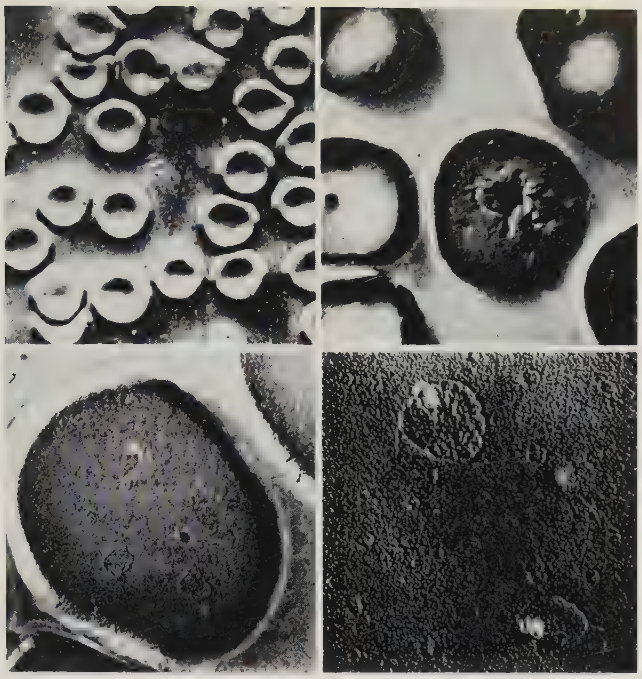


Fig. 393. Electron micrographs of normal red blood cells showing craterlike depressions. Although they may be artifacts, they are related to the integrity of the red blood cells; e.g., after exposure of the cell to antierythrocytic antibodies these craters become wider, deeper, and more numerous. (From Bernard, J., and Bessis, M.: Hematologie clinique, Paris, 1958, Masson & Cie.)

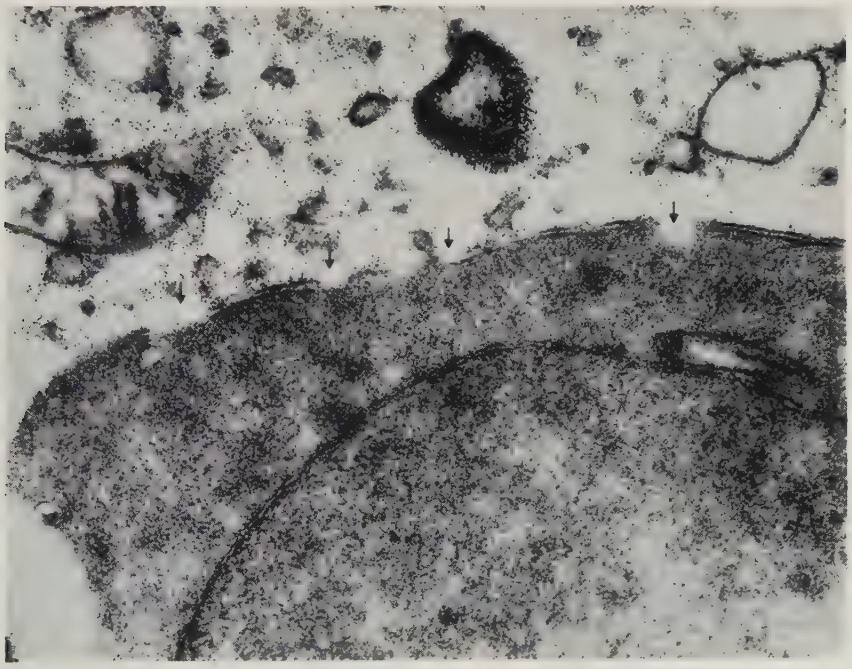
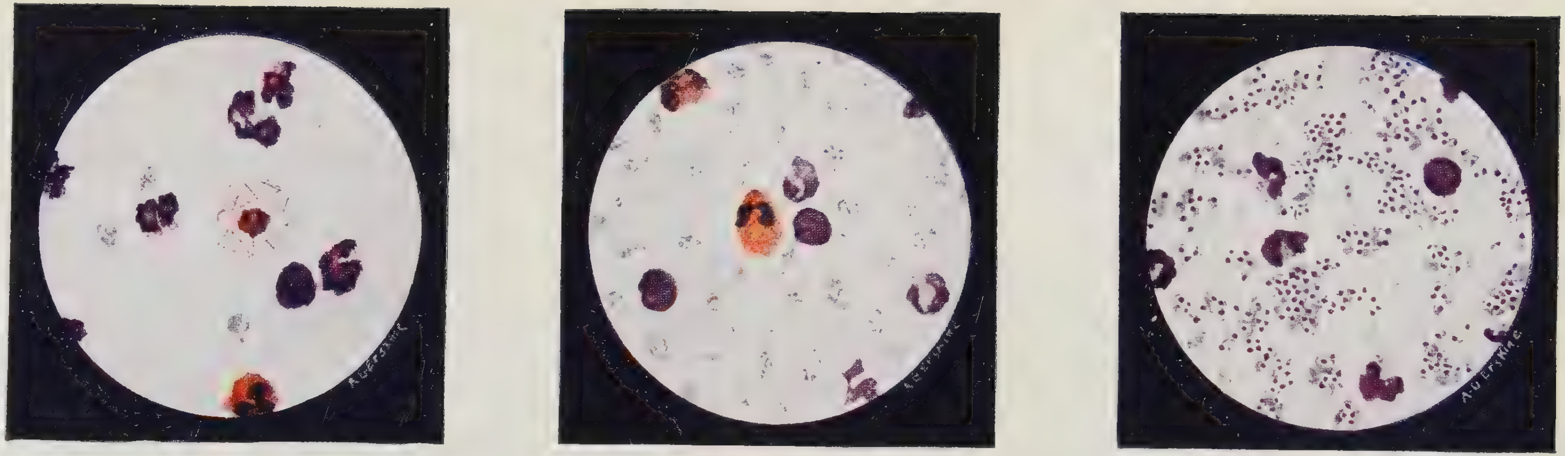


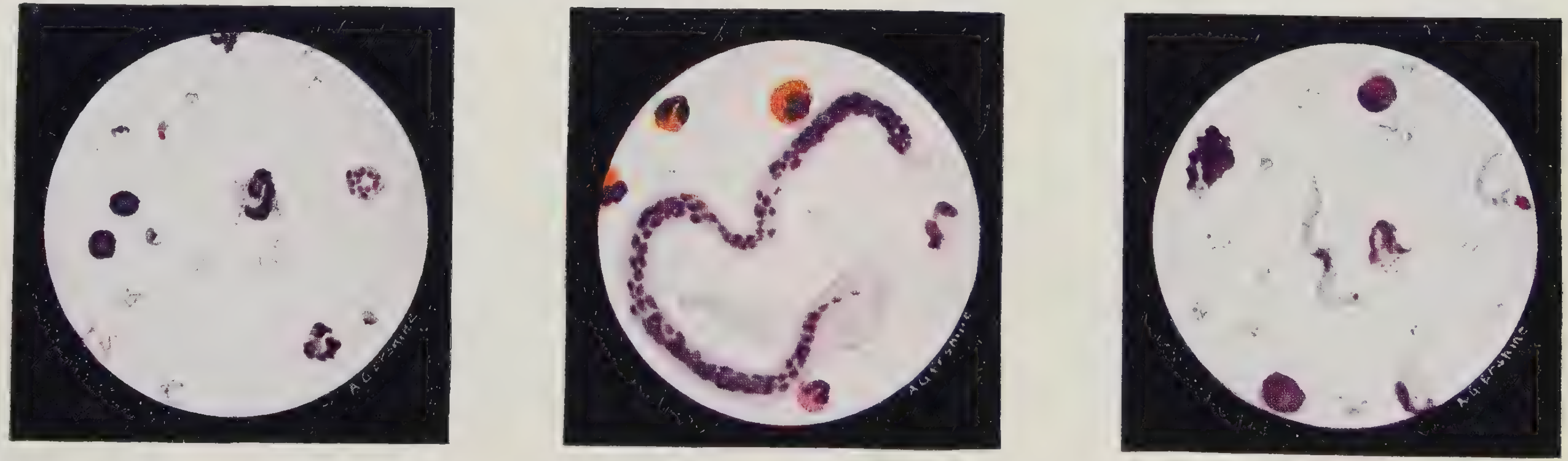
Fig. 394. Electron micrograph of pinocytic transfer of ferritin molecule from reticulum cell to erythroblast. Cell membranes between the 2 cells disappear and small depressions form (arrows). (Courtesy Dr. M. Bessis, Paris, France.)



Normal thick drop
(×950)

Lead poisoning
(×950)

Plasmodium falciparum in fatal tropical malaria
(×950)



Malarial parasites
(×950)

Filariasis bancrofti
(hematoxylin-Giemsa stain)

Trypanosomiasis gambiense
(×950)

Fig. 395. Thick drops of blood. (Giemsa stain.)

in the spread. The reason for this is that every field in the thick drop preparation is equivalent to 50 fields in the spread. Thus examining 4 fields in the thick drop is equal to examining 200 fields in the spread. The erythrocytes are dissolved by the water in the diluted Giemsa stain, making it easy to see blood parasites and structures of young erythrocytes. The eosinophils are very definite, the granules being bunched together. Basophils appear as a group of fibrin needles around a circular solidly stained center, the whole structure being stained purple-red. Neutrophils are typical, except that the cytoplasm has dissolved. The nuclei and granules, however, remain. In other words, all substances in the blood that are soluble in water are dissolved in the thick drop staining process. The other structures remain as they are.

Procedure:

1. Place 2 drops blood on a glass slide.
2. Spread the 2 drops in a circle to 3 times the original diameter of the drops.
3. Allow to dry thoroughly either in an incubator (after preliminary drying in the room) for ½-3 hr. or at room temperature. Do not leave it in bright sunlight or anywhere where dust may settle on the slide. **Do not fix.**
4. Place the slide on a staining bridge. Gently cover with Giemsa stain, taking care not to dislodge the drops from the slide.
5. Allow the diluted stain to remain in contact with the drops of blood for 3 min. or until the hemoglobin begins to dissolve. This can be detected by holding a piece of white paper under the slide. The hemoglobin will be seen flowing through the diluted stain. Then gently tilt the slide and allow the stain to drain off. Re-cover with diluted Giemsa stain, taking care not to dislodge the drops of blood.
6. Stain for 30 min.
7. Remove the stain by gently tilting the slide.
8. Wash very carefully with neutral distilled water so as not to remove the blood drops from the slide and allow to dry by standing the slide on end. **Do not blot.**
9. Examine with the oil immersion objective.

Modified method of staining thick drops for examination for malarial parasites[234]*

1. Mix Giemsa stain with buffer solution pH 7.0-7.2 or neutral distilled water in the proportion of 1 drop Giemsa stain for each 2 drops buffer. Mix in a test tube or small cylinder.
2. Place just enough stain on the thick drop to cover it and allow to remain for 3 min.
3. Wash either by immersing in a jar of neutral distilled water or in the usual manner of washing a slide.

Combined thick drop-thin film method (Agress modification)

1. Place a drop of blood on one end of a glass slide and spread to about 2 or 3 times its diameter in a circular manner, using the puncture point or the corner of another glass slide.
2. Make a thin spread of blood on the rest of the slide in the standard manner.
3. Separate the area of the thick drop and the thin spread by drawing 2 or 3 lines between them with a wax pencil.
4. Either place the slide in a 37° C. incubator for ½ hr. or allow to remain at room temperature for 1 hr.
5. Add Wright stain for 2 min. to the thin spread **only.**
6. Dilute with neutral distilled water or buffer for 2 min., taking care that this stain does not run over onto the thick drop.
7. Pour off.
8. Flood the entire slide including the thick drop with diluted Giemsa stain (freshly prepared, using 1 drop stock Giemsa stain to 3 ml. neutral distilled water or buffer solution).
9. Allow to remain on the slide for 30 min.
10. Wash and air dry.

Direct count of eosinophils in blood

The routine differential count is usually sufficient to determine the relative number of eosinophils in the blood. On the basis of the total white cell count and the differential count, the number of eosinophils per c.mm. can be calculated, but for certain clinical purposes an absolute eosinophil count made in a counting chamber is preferable. There are 2 types of diluting fluid. An example of one is the Friedman solution which contains eosin to stain the granules, acetone as a fixative of leukocytes, and distilled water to lyse the erythrocytes. The second type, which is usually a modification of the Randolph fluid,[236] uses eosin or phloxine as acid dye, propylene glycol to lyse the red cells, sodium carbonate as accentuator, and heparin to prevent clumping of the white cells.

Method of Friedman[235]

Reagent:

Aqueous eosin, 1%	5 ml.
Acetone	5 ml.
Distilled water	100 ml.

Procedure:

1. Dilute the blood in the usual manner in a white blood counting pipet, using the Friedman reagent as the diluting fluid. The diluting fluid stains only the eosinophilic cells, which are easily distinguishable with the high dry power lens of the microscope. The unstained leukocytes appear as gray bodies in this diluting fluid.
2. Count the eosinophils in all 9 large squares of

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*This is the method used at the Ryder Memorial Hospital, Humacao, Puerto Rico.

2 chambers and repeat the count with a second pipet.

Calculations: See method of calculation described for Method 2.

Method 2

Reagent:

Propylene glycol	50 ml.
Distilled water	40 ml.
Aqueous phloxine, 1%	10 ml.
Aqueous sodium carbonate, 10%	1 ml.
Heparin sodium	100 units

Procedure:

1. Draw blood to mark 1 (top of the stem) in a white counting pipet.
2. Draw the diluting fluid into the pipet until a total of 11 vol. is reached. The dilution of the blood is 1:10.
3. Shake well.
4. Let stand for 10-15 min.
5. Again shake well, let out a few drops, and fill both sides of the counting chamber as usual.
6. Let stand in the counting chamber for 3 min.
7. Count under the low power with the condenser in place. Adjust the light by **slightly** lowering the condenser until the maximum number of white cells is seen and the eosinophils appear as very tiny red stained cells. The eosinophilic granules are brilliant red.
8. Count all the eosinophils in the entire 9 fields of the counting chamber (9 sq.mm.).
9. Count the eosinophils in the counting chamber on the other side of the hemocytometer in the same way and calculate the average of the two counts.
10. Multiply the average by 11 to obtain the number of eosinophils in 1 c.mm. blood. (Dilution of blood is 1:10, area counted is 9 sq.mm., depth of the counting chamber is 1/10 mm. The volume counted is $1/10 \times 9 \times 1/10 = 9/100$ or approximately 1/11 c.mm.).

To obtain per cent of eosinophils: The % of eosinophils is the eosinophil count per c.mm. divided by the white blood cell count per c.mm.

Report the count made in the counting chamber as the absolute count and the percentage as indicated.

Example:
Patient's white count: 8163/c.mm.
Eosinophils: 276/c.mm.
276 : 8163 = 0.03 or 3%.
Normal: 2-4%; 150-300/c.mm.; average, 200.

Thorn test

The blood eosinophil response to the injection of pituitary adrenocorticotropic hormone (ACTH) or epinephrine is the basis of the Thorn test[237] of adrenal cortex function. In normal subjects, such injections are followed in 4 hr. by a fall in the total blood eosinophil count of over 50%. In patients with Addison's disease, the decrease in eosinophils is 20% or less. In patients with adrenal insufficiency secondary to pituitary disease, the injection of epinephrine is followed by no significant change in the eosinophil count, but injection of ACTH produces a normal decline in eosinophils.

This screening procedure is performed with the patient starting in the fasting state and remaining so throughout the 4 hr. period of observation. A total blood eosinophil count is performed, and then 25 mg. ACTH or 0.3 ml. 1:1000 dilution of epinephrine is injected intramuscularly. Four hours later another eosinophil count is performed, and this value is compared with the original count.

This test is merely a screening test. An attempt is made to stimulate the adrenal cortex by administration of either epinephrine or adrenocorticotropic hormone. Changes in the circulating eosinophil count are accepted as indicators of adrenal cortical function. After the intramuscular administration of 0.3 ml. epinephrine hydrochloride, the healthy anterior pituitary releases adrenocorticotropic hormone (ACTH) which stimulates the adrenal cortex, thus increasing the output of its steroids. A maximal decrease of circulating eosinophils will be noted 4 hr. after the injection. If the pituitary pathway is not to be included, ACTH may be administered directly, either 25 units I.M. or 50 units in 500 ml. normal saline intravenously over a period of 8 hr. This procedure may be repeated the next day. The responsive adrenal will increase its steroid output.

In addition to the eosinophil count, the urinary corticoids before and after ACTH or epinephrine administration should be examined. The most important of these corticoids are the 17-hydroxysteroids.

Chapter 63

Coagulation of blood and hemorrhagic diseases

JOHN D. BAUER

Hemostasis, the arrest of bleeding, depends on 2 main factors: structurally and functionally intact blood vessels and adequate blood coagulation. Inadequate blood vessels are more frequently the cause of bleeding than defects in coagulation. The onset of bleeding is always associated with a faulty vascular system, whereas a defective clotting mechanism is responsible for the continuation of the bleeding. The patient with afibrinogenemia, whose blood does not clot, does not bleed until a blood vessel is injured. The freely flowing blood within blood vessels is the result of an equilibrium established by the various clotting factors and their antagonists. Coagulation defects occur when there is a decrease in coagulation factors or an increase in inhibitors (hypocoagulability), whereas an increase in coagulation factors and a decrease in inhibitors may lead to hypercoagulability. This equilibrium between coagulation factors and inhibitors is of the greatest importance in the production of thrombosis of blood vessels, a phenomenon which in its importance by far exceeds the problem of bleeding tendency.

THEORIES OF BLOOD COAGULATION AND LABORATORY ASPECTS OF HEMOSTASIS

Historical review

The ferment theory was first advanced by Alexander Schmidt.[238] He emphasized that the specific ferment for blood coagulation was thrombin, a product which occurred with the breaking down of the corpuscular elements of the blood, especially the leukocytes. A schematic representation of the Schmidt ferment theory is shown in Fig. 396.

It was Hammarsten[239] who introduced the question of the role of fibrinogen into blood coagulation. Arthus and Pages[240] first called attention to the calcium content of the blood as of prime importance in blood clotting. They noted that the addition of oxalates to blood, which made it calcium-free, prevented clotting and that the re-addition of calcium produced clotting. Pekelharing[241] made further researches into this calcium factor. Hammarsten claimed that the presence of calcium is necessary before prothrombin could be activated into thrombin. Fuld[242] and Spiro[243] showed that 3 substances, which are precursors of thrombin, are necessary for blood clotting: namely, the thrombogen of Morawitz, the plasmochyme of Fuld, and soluble calcium salts. Another substance which Morawitz introduced into the literature was thrombokinase, which Fuld called cytozyme. Fig. 397 illustrates Morawitz' ideas on blood clotting.[244]

Howell[245-249] is responsible for the present-

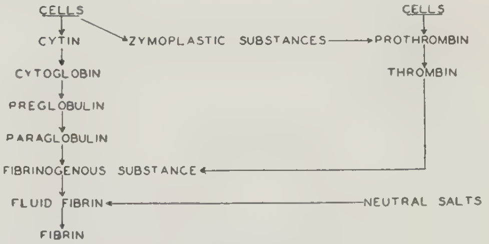

Fig. 396. Schmidt ferment theory

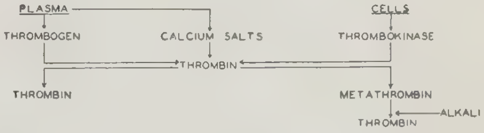

Fig. 397. Morawitz blood clotting theory.

day understanding of the phenomenon of blood coagulation.

Prothrombin + Ca ions + Thromboplastin = Thrombin
Thrombin + Fibrinogen = Fibrin

Within the past 20 years, interest in blood coagulation has been greatly stimulated, and as a result a voluminous literature concerning hemostasis has been accumulated. As new facts have been disclosed, theories of blood coagulation have been modified and amplified.

Present concept of coagulation

The ultimate aim of blood clotting is the formation of fibrin. The fibrin formation is accomplished by the interaction of 2 systems—an intrinsic system, in which only factors present in the blood itself take part, and an extrinsic system, in which tissue factors, such as tissue thromboplastin, also take part.

The external system which has to do with the initiation of the clotting mechanism does not require platelets and factor VIII but needs factor VII and factor V. The intrinsic system which is responsible for the definite hemostasis does not require factor VII but needs all other factors which take part in phase 1 of clotting. Both systems require factor V and Stuart-Prower factor and calcium. After the formation of 1 or 2 intermediate products (blood and tissue convertin ?), both processes, the intrinsic and the extrinsic, follow a similar pathway to the formation of fibrin.

The clotting factors are enumerated in Table 240.

Table 240. *Clotting factors**

Assigned number of clotting factor	*Synonymous names*
I	Fibrinogen
II	Prothrombin
III	Tissue thromboplastin
IV	Calcium
V	Labile factor; accelerator globulin; proaccelerin
VI	Accelerin; accelerator globulin
VII	Stable factor; proconvertin
VIII	Antihemophilic factor; AHF Antihemophilic globulin; AHG Thromboplastinogen
IX	Plasma thromboplastin component; PTC Christmas factor
X	Stuart factor; Stuart-Prower factor
?	Plasma thromboplastin antecedent; PTA
?	Hageman factor; HF
?	Platelet factors 1-8, such as serotonin, antifibrinolysin, retraktozyme, fibrinogen and factor V-like substances, antiheparin, and thromboplastinogenase

*From Bauer, J. D., Toro, G., and Ackermann, P. G.: Bray's clinical laboratory methods, St. Louis, 1962. The C. V. Mosby Co.

Coagulation can be visualized as occurring in 3 stages. The first phase is concerned with the production of plasma thromboplastin. In this reaction, the following factors are involved: platelets (mainly platelet factor 3), plasma factors VIII and IX, PTA factor, Hageman factor, Stuart-Prower factor, Quick's erythroid factor, and calcium. Spittel recently described a temporary pathologic plasma factor which has an accelerating influence on plama thromboplastin formation, and if it is confirmed, it may be of considerable clinical importance. The intrinsic thromboplastin is labile and disappears within 1 hr. In the process of clotting, factors VIII and V are consumed and may thus be considered to be substrates for enzymes. Factors IX and VII are not consumed and are probably the enzymes acting on the substrates just mentioned.

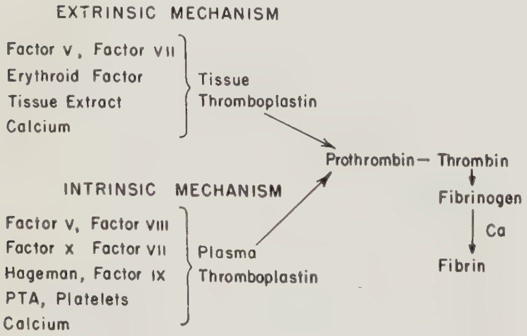

Fig. 398. Diagram of blood clotting. (From Bauer, J. D., Toro, G., and Ackermann, P. G.: Bray's clinical laboratory methods, St. Louis, 1962, The C. V. Mosby Co.)

The second stage of coagulation is the conversion of prothrombin to thrombin through the action of plasma thromboplastin generated in the first stage. For the conversion of prothrombin to thrombin by tissue thromboplastin, a phospholipid (probably cephalin), calcium, and factors VII and V are necessary. The nature of the conversion of prothrombin to thrombin is not known but probably involves splitting off portions of the original prothrombin molecule. Thrombin is normally not present in the circulation.

The third phase is the conversion of fibrinogen to fibrin with the help of thrombin produced in the second stage. The result of fibrin formation is the clotting of blood. Thrombin, a proteolytic enzyme, splits off glutamic acid containing portions of the fibrinogen molecule. In the freely flowing blood the stability of fibrinogen, a protein of high molecular weight, in solution is apparently due to negatively charged terminal groups which include glutamic acid. After the glutamic acid-containing portions are split off, the remaining segments of the original fibrinogen molecule are called fibrin molecules. These fibrin molecules are attracted to each other by electrostatic forces and polymerize by end-to-end and side-to-side apposition to produce visible fibrin fibers. After the fibrin clot is formed, it retracts, and after a certain period of time it is dissolved by the fibrinolytic system of the blood. The clot retraction is essentially a function of platelets which undergo viscous metamorphosis, thus apparently releasing a factor called retraktozyme.

According to recent investigators,[250] glucose, divalent cation, and a critical pH are also required.

Each phase is subject to the action of inhibitors. These substances which help to maintain the blood in liquid state are of great physiologic importance. They are responsible for the disappearance of coagulation factors which may be formed in excess. If they occur in abnormal amounts or types, they may lead to a bleeding tendency.

Inhibitors of phase 1: Antithromboplastin (Tocantins), antifactor VII factor, when present in excess is thought to be responsible for some cases of hemophilia. A circulating anti-tissue-thromboplastin may also exist (Stefanini and Dameshek). This will be discussed in the section on immunohematology.

Circulating inhibitors may be the result of transfusions, pregnancies, or allergies or may accompany certain diseases such as collagen diseases. In phase I circulating anticoagulants may prevent the formation of thromboplastin, but they do not act on already formed thromboplastin or thrombin. Inhibitors to factor VII and factor V have been described. The activity of heparin, which is mainly an antithrombin, also extends into phase I since it has some antithromboplastic and antiplatelet action (serotonin).

Inhibitors of phase 2: Several types of antithrombin occur normally in the plasma and serum. Antithrombins V and VI have been considered responsible for bleeding tendencies. Heparin, a mucopolysaccharide produced by blood and tissue basophils (mast cells), acts as an antithrombin by combining with a heparin cofactor and thrombin (antithrombin II). Fibrinogen (antithrombin I) by its ability to adsorb thrombin also acts as an antithrombin. Seeger antithrombin III acts without the help of heparin.

Inhibitors of phase 3[251, 252]**:** The main inhibitor is fibrinolysin, the end product of a fibrinolytic enzyme system. The naturally occurring fibrinolytic enzyme precursor is called plasminogen. In the presence of activators (kinases) it is converted into a trypsinlike proteolytic enzyme, fibrinolysin, which hydrolyzes fibrin at neutral pH. The kinases involved may be naturally occurring such as tissue kinases, trypsin, plasmin, and urobinase (urine), or they may be bacterial in origin such as streptokinase and staphylokinase.

Properties of clotting factors

Most clotting factors are alpha and beta globulins, produced by the liver. They have a half-life in the circulating blood of from a few hours to a few days. As will be shown later, deficiency in clotting factors may be either congenital or acquired.

Factor I (fibrinogen): Fibrinogen is a soluble circulating protein which is not present in serum and is precipitated by 23-45% ammonium sulfate. In the electrophoretic field it moves with the beta and gamma globulins. It is formed in the liver and probably in the bone marrow and is the precursor of fibrin. A level below 100 mg./100 ml. plasma is indicative of hypofibrinogenemia. The congenital or acquired absence or the diminution of fibrinogen will be considered in the discussion of hemorrhagic diseases. Fibrinogen is increased in several conditions such as pregnancy, infectious diseases, myocardial infarction, rheumatic fever, and old age.

Factor II (prothrombin): Prothrombin is a glycoprotein with alpha globulin motility in the electrophoretic field. It is precipitated by 50% ammonium sulfate and is normally present in plasma. It is consumed in the clotting process so that the normal serum contains only a trace. Vitamin K_1 is essential for the production of prothrombin by the liver and perhaps by the lung. Vitamin K_1 is a coenzyme which the liver utilizes for the formation of prothrombin and factor VII. Acquired and congenital hypoprothrombinemia are included in the discussion of hemorrhagic disorders.

Prothrombin is converted to thrombin by the action of thromboplastin. In this process it loses 2 amino acids and its carbohydrate fraction. The most common prothrombin deficiency is produced therapeutically by the administration of Dicumarol, which is a powerful anticoagulant.

The structural formulas of Dicumarol and vitamin K_1 are so similar that it is felt that Dicumarol acts by replacing the prosthetic group of the hepatic enzyme which utilizes vitamin K_1 in the production of prothrombin and factor VII. Administration of Dicumarol produces factor VII deficiency and to a lesser degree depression of prothrombin and factors IX and X. The effect of Dicumarol administration can be counteracted by the administration of vitamin K_1 (liver function must not be impaired). Because of the described action of Dicumarol, patients on this type of anticoagulant therapy will show not only an abnormal 1-stage prothrombin time, but also an abnormal thromboplastin generation test. As Dicumarol is detoxified in the liver and excreted by the kidneys, diseases of these 2 organs will markedly alter the results of Dicumarol therapy.

In the first week of life the prothrombin level is low (even more marked in premature babies—danger in circumcision).

Factor III (thromboplastin): Thromboplastin is required for the conversion of prothrombin to thrombin. We distinguish 2 types, as indicated in Fig. 398: (1) tissue thromboplastin, which acts in seconds and requires for its formation tissue extract, probably phospholipids, and factors V, VII, and X, and (2) plasma thromboplastin, which requires several minutes for production and needs the following factors: Hageman, V, VIII, IX, X, PTA, platelets, and calcium. Both thromboplastins can be activated by plasma and serum.

Factor IV (calcium): Calcium ions, though admittedly in very low concentrations, are necessary for the formation of thromboplastin. In vitro, the removal of calcium is the basis of the most commonly used laboratory anticoagulants. In vivo, the removal of calcium must be counteracted if citrated blood is used instead of heparinized blood in massive transfusions or in exchange transfusions.

Factor V (labile factor): Factor V is labile and is activated by thrombin to factor VI. It is consumed during clotting and is not affected by Dicumarol. Inactivated by heating to 58° C., it is precipitated by 33% ammonium sulfate. It is a proenzyme, activated by a plasma factor, and functions as a thromboplastin cofactor. It is lost in stored oxalated plasma and is not found in serum.

Factor VI (accelerin): Factor VI is a tissue thromboplastin cofactor and is produced either in the liver or during the conversion of prothrombin to thrombin.

Factor VII (stable factor): Factor VII is a plasma factor which is not lost in stored oxalated plasma. It is not consumed during clotting and is thus present in serum. It is produced in the liver with the aid of vitamin K_1. It is often deficient in the neonatal period. Factor VII is affected by the administration of Dicumarol and related drugs.

Factor VIII (antihemophilic factor): Factor VIII is a beta globulin which is stable for 5 min. at 38°-49° C. at a pH of 6.8. It is produced by the reticuloendothelial system and is necessary for thromboplastin formation in plasma. Factor VIII is probably produced in the liver, lungs, and kidneys, and its production is related to the hemophilia chromosome (sex-linked). In the absence of factor VIII, platelet factor I may be part of an alternate pathway of activation of prothrombin. Factor VIII is consumed during the clotting process (see discussion of hemophilia and hemorrhagic disorders) and is thus not present in serum. Though labile it can be preserved in citrated plasma at 20° C. and in lyophilized form.

Factor IX (plasma thromboplastin component): This factor occurs in plasma and serum, probably in an inactive form.

Factor X (Prower-Stuart factor): This factor is similar to factor IX and is involved in thromboplastin production. It is present in plasma and serum.

Plasma thromboplastin antecedent (PTA): This is a factor present in plasma and serum and is stable during blood storage.

Hageman factor: This factor, which is activated by glass, is necessary for the development of blood thromboplastin. It may also have some relationship to capillary permeability.

Platelets: Platelets are involved in the initiation of blood clotting, which is considered in the discussion of the mechanism of coagulation.

Mechanism of coagulation

After injury the smaller blood vessels contract and retract. The contraction is aided by platelets which release a vasoconstricting agent, serotonin. The release of this and other platelet factors is apparently related to viscous metamorphosis of platelets. The endothelial lining of the blood vessels becomes sticky so that opposing surfaces adhere to each other. The endothelial change may be related to electrostatic changes so that the negative endothelial cells attract positive platelets. There may well be several ways by which blood clotting is initiated. The platelets which show viscous metamorphosis become sticky and adhere to the vessel wall. A plasma factor may be helpful in the production of this stickiness and adhesiveness of the platelets. On the other hand, Quick believes that a minute amount of fibrin may activate the plasma factor, which in turn acts upon the platelets so that they disintegrate. At one time factor VIII was believed to be the activating plasma factor. Quick describes an erythrocytic clotting factor which is released when the red cells come in contact with a foreign surface.

The initiation of clot formation may be related to the release of tissue thromboplastin at the time the blood vessel is injured. Thromboplastin reacts with prothrombin to form a small amount of thrombin which in turn will not only produce a small amount of fibrin, but will also act upon platelets, stimulating them to release platelet factors. On contact with glass the interaction of the Hageman factor and the Quick erythrocytic factor apparently initiates clotting. Immediately after injury, the external system of blood clotting is called into play, and whether an intermediate product (such as convertin) is involved or not, there is a slow conversion of prothrombin to thrombin by tissue thromboplastin. The small amount of thrombin thus formed activates factor V and in the presence of calcium the conversion of prothrombin to thrombin. As this process continues, the production of thromboplastin increases in speed. Thrombin then acts on fibrinogen to produce fibrin. This process is dependent on the concentration of fibrinogen, on the pH, on the temperature, and probably also on platelet action.[254] It is evident that coagulation is the result of interplay of actions and counteractions so that at each step the rate of production of the clotting product must exceed the rate of formation of the inhibitor. Coagulation thus consists of an action not unlike that of a pinwheel in a Fourth of July fireworks display. There is the slow starting outer phase (thromboplastin to minimal prothrombin conversion), a sputter (thrombin formation), a high-speed brilliant phase with sparks flying in all directions (thrombin activating accelerators, thrombin labilizing platelets, more prothrombin being converted, fibrinogen being converted to fibrin), and then the slowly dying-out phase (a firm clot being formed and fibrin slowly applying the brakes).

TESTS FOR HEMORRHAGIC DISEASES

The investigation of the bleeding patient must include (1) good history with special attention to previous bleeding episodes, allergies, infections, family history, and liver disease, and (2) inspection and examination of the patient with special attention to the skin, to the presence of petechiae (thrombocytopenia), ecchymoses (coagulation defect), and to mucous membranes such as gums. The size of the liver, spleen, and lymph nodes may offer possible clues to the disease.

As will be discussed later, the hemorrhagic diseases include 3 main groups of defects: (1) vascular defects, (2) coagulation defects, and (3) platelet defects. In order to evaluate all 3 and despite some overlap, coagulation tests should follow a given order:

1. Tests for vascular function and capillary permeability, such as bleeding time and tourniquet test.
2. Tests for platelet function, such as clot retraction, platelet count, and bleeding time.
3. Tests for over-all clotting ability of the blood, such as clotting time.
4. Tests for phase III of the clotting mechanism, such as fibrinogen level.
5. Tests for phase II of the clotting mechanism, such as plasma prothrombin time.
6. Tests for phase I of the clotting mechanism, such as serum prothrombin time, and thromboplastin generation test.
7. Tests for circulating anticoagulants.

Collection of blood

General instructions for all coagulation tests: As in all laboratory tests, strict adherence to technic in every detail is necessary. Most tests should be performed at 37° C., the body temperature. Carefully cleaned, not scratched, 13 × 75 mm. test tubes are used in almost all the procedures. Pipets should be acid cleaned. Delays in completing the test should be kept at a minimum, as contact with glass tends to shorten the clotting process and standing at room temperature will destroy the thermolabile factors.

Siliconized glassware: Frequently in the following tests siliconized glassware will be mentioned.

Coating with silicone: The glassware must be clean, must be kept from dust when siliconized, and must be stored and cleaned separately from the rest of the laboratory glassware. For siliconizing, a mixture of 1 part Drifilm* and 4 parts petroleum ether are used.

Test tubes:

1. Fill 1 tube with silicone mixture to the top and then pour the mixture from 1 tube to the other, thus coating the tubes.
2. Fill coated tubes with distilled water and allow to stand for 5 min.
3. Rinse with distilled water 3 times.
4. Dry in an oven at 120° C. for 1 hr.

Adequacy of siliconization can be checked by half filling the tubes with water and observing the convex meniscus.

Pipets:

1. With a rubber bulb draw silicone mixture to a point close to the mouth end.
2. Empty.
3. Rinse with distilled water 3 times.
4. Dry in an oven at 120° C. for 1 hr.

Syringes: Coat the barrel by aspirating 3 ml. silicone mixture. Hold the syringe with the tip directed upward and pull the plunger up and down 3 times. Rinse the barrel and plunger repeatedly and dry separately in an oven at 120° C. for 1 hr.

Needles: Silicone coating of needles is not usually considered necessary if new needles are used for each puncture. If coating is required, the silicone mixture is aspirated through the needle and is then blown out by compressed air. Immerse the needle in ammonium hydroxide, dry, and clean the bore with a nylon bristle.

For special coagulation test, apply 6 coats of silicone and polish the siliconized surfaces after each coating with a nylon cloth.

Removal of silicone:

1. Wash glassware in warm water and then immerse in the following solution overnight.

Acetone, technical	2 L.
Distilled water	2 L.
NaOH (solid)	150 gm.

2. Dissolve the sodium hydroxide in about 300 ml. water before adding to acetone and water.
3. After immersion, rinse well and dry in an oven.

TESTS FOR STATUS OF CAPILLARIES

BLEEDING TIME

Method I—Duke[255]

Procedure:

1. Cleanse the ear lobe and make a cut, not a puncture, with a scalpel blade, needle, or Steri-Lancet, 3-4 mm. deep and approximately 5 mm. long, against a microscope slide held behind the ear lobe.
2. Touch a piece of blotting paper to the area every 30 sec., touching only the head of the drop. The test is terminated when no more free drops can be picked up on the blotting paper.

The bleeding time is measured from the moment the blood appears to the cessation of bleeding. Record the time with a stopwatch.

Normal: 1-3 min.

Method II—Ivy[256]

Materials:

1. Sphygmomanometer.
2. Sharp-pointed blade.

Procedure:

1. Place a blood pressure cuff on the arm and inflate to 40 mm. Hg.
2. Cleanse the volar surface of the forearm below the cuff and make a skin wound 3-4 mm. deep.
3. Blot the incision every 10 sec. until there are no more free drops, maintaining a pressure of 40 mm. throughout the procedure.

The bleeding time is measured from the moment the blood appears to the cessation of bleeding. Record the time with a stopwatch.

Normal: 0-4 min.[257]

Method III—Copley-Lalitch[258]

Procedure:

1. Cleanse the finger and make a puncture wound 6 mm. deep.
2. Immerse the wound in sterile physiologic saline warmed to 37° C. until there is no free flow of blood.

The bleeding time is measured from the moment of the wound to the cessation of bleeding.

Normal: In 95% of persons the normal values are less than 3 min. with a range of 170-340 sec.

Comments on skin bleeding time

The response of the skin capillary bed as measured by these tests is dependent upon many factors, such as skin turgor, adiposity and vascularity of subcutaneous tissue, and variations in technic, as well as upon the state of the blood vessels and the blood coagulation mechanism. Obviously, any test which attempts to measure so many variables can give information only of a general nature and serves only as a gross screening test.

Abnormally prolonged bleeding times are associated with thrombopenic states.

In a discussion on the mechanism of hemostasis, Macfarlane[259] called attention to the lack of capillary contractility associated with thrombocytopenic purpura. By visualization of the nail beds under the microscope, he noted differences in the capillary reaction in various conditions and particularly called attention to the abnormal reaction in thrombocytopenia. This reaction of capillaries is of such marked degree that it might be considered an integral part of the disease process. The extent of bleeding manifestation in cases of thrombocytopenic purpura is as directly related to the state of the capillary bed as it is to the platelet level. Although bleeding times and platelet levels usually parallel one another, there are cases in which the bleeding time may be prolonged, with only a slight reduction in platelets, and in other cases the reverse may be true. It has been suggested that an antigenic relationship exists between platelets and vascular endothelium with a common etiology for both.[260]

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*No. SC-87; General Electric Silicones, Waterford, N. Y.

Another defect in the clotting mechanism associated with prolonged bleeding time is hypoprothrombinemia. The experience of surgeons prior to the discovery of the value of vitamin K therapy amply attests to the capillary bleeding in patients with obstructive jaundice.

A surprising relationship between a severe abnormality in the coagulation mechanism and a normal bleeding time test exists in hemophilia. With the minor wound inflicted in the test, the capillaries are sufficiently resilient to shut off immediate bleeding. However, it is not at all uncommon for bleeding to return at the puncture site an hour or more later. Occasionally such recurrent bleeding may be sufficiently severe to cause concern. If hemophilia is strongly suspected, laceration of the skin should be zealously avoided, and punctures for tests should be limited only to those necessary for diagnosis and treatment. The bleeding time is an unnecessary test in this condition. In other diseases simulating hemophilia, such as deficiency of the 2 plasma thromboplastin factors, plasma thromboplastin component (PTC) and plasma thromboplastin antecedent (PTA), Rosenthal[261] reported usually normal bleeding times.

There exists a nondescript group of hemorrhagic diatheses, most of which have appeared in the older literature, associated with a prolonged bleeding time. Among these might be mentioned the hereditary capillary fragility group, variously labeled pseudohemophilia A, von Willebrand's disease, and purpura of capillary weakness. Glanzmann's thrombasthenia is another type in which some unknown defect of platelets, which are normal in numbers, is reputedly responsible for abnormal bleeding. It is probably the same disease that von Willebrand described. Pseudohemophilia B also falls into this group of diseases with prolonged bleeding time. As our knowledge of the clotting mechanism is elaborated, this group becomes more circumscribed.

Bleeding time may be prolonged in elderly individuals in whom no defect in clotting mechanism exists. Such abnormal bleeding is purportedly related to lack of elasticity of capillaries in the aged, though bleeding time does not measure capillary fragility (hyperpermeability) but rather response of capillaries to injury.

Another parodoxical relationship exists in which purpura (nonthrombocytopenic) is pronounced, capillary fragility tests are markedly abnormal, and yet the bleeding time tests are usually normal. Some of the allergic purpuras (Schönlein-Henoch) fit into this category as do those related to sepsis, uremia, and vitamin deficiencies.

CAPILLARY FRAGILITY TESTS

Capillary fragility tests can be roughly divided into 2 principal groups—those applying pressure and those applying suction. Many modifications, aimed chiefly at standardization of the technic, have been devised.

Method of Rumpel-Leede[262] (tourniquet test)

This test, which originally consisted in placing a tourniquet about the arm and observing the number of petechiae in the area distal to it, has had so many modifications of time of application and levels of pressure that authorship of most of the modifications can hardly be traced. In general, the tourniquet has been replaced by a blood pressure cuff. There is no agreement as to whether the blood pressure should be midway between diastolic and systolic pressures or whether 100 mm. Hg is the desirable level. The time of application is variably suggested as 5-10 min. There is some difference as to whether the entire extremity should be examined for petechiae or whether the number in a limited area should be counted.

There is also no unanimity of opinion concerning normal values. In general, it can be said that most **normal individuals** show relatively few petechiae, perhaps 10 or less. Those with a marked capillary fragility show many petechiae, sometimes almost covering the entire skin. We should like to add a few comments based on our experience with these modifications.

It is not at all uncommon for a band of petechiae to appear at the lower edge of a tourniquet or blood pressure cuff where the skin has been pinched. We suggest, therefore, that this area be disregarded in an appraisal of the results. Another observation is that it is not at all uncommon for petechiae to appear several minutes after the application of pressure or suction. For this reason, our routine procedure in any capillary fragility test is to include a re-examination of the area at least 30 min. after the application of pressure or suction.

Method of Quick[263]

1. Apply blood pressure cuff to the upper arm and inflate to a pressure midway between systolic and diastolic levels.
2. Maintain pressure for 5 min.
3. Release cuff and inspect arm, wrist, and hand for petechiae.

Normal: Up to 5 petechiae.*

Method of Gothlin[264]

1. Carefully inspect the forearms for petechiae or other marks which might be mistaken for them.
2. Mark off in ink an area 6 cm. in diameter in the antecubital fossae on each upper extremity.
3. Apply blood pressure cuffs to both arms and inflate simultaneously to 35 mm. Hg, maintaining this pressure for 15 min. with the subject resting the forearms on a table.
4. Release the pressure and inspect the outlined area for petechiae, using a magnifier and bright light.
5. Multiply the number of petechiae in both arms by 2.

If the **Gothlin index** is 8 or less, the result is considered normal; if more than 12, capillary fragility is considered abnormal. If the value falls between 8 and 12, the results are equivocal, and the following step is suggested.

6. Not before 1 hr. and preferably the next day or later, repeat the procedure, with the pressure at 50 mm. Hg for 15 min. Note the number of petechiae. Do not double this number, as in step 5, but add it to the results of step 5.

If the sum of steps 5 and 6 exceed 12, the result is considered abnormal.

Normal: 0 to 12 petechiae (**Gothlin index**).

Petechiometer method†

The apparatus consists of a plastic suction cup† 2 cm. in diameter, with a built-in spring-activated suction piston. A simple locking device enables adjustments of negative pressures at 10, 20, and 30 cm. of negative pressure. A built-in magnifying lens enables visualization of the area subjected to the test. The kit also contains a plastic circular disk the size of the suction cup, with the central area marked off by a circle 1 cm. in diameter.

1. Inspect a nonhairy skin area in either the subclavicular fossa or the inner aspect of the arm for blemishes which might be mistaken for petechiae. Note and eliminate from the counts.
2. Rim the suction cup with lubricant to establish an airtight seal and then apply it to the skin at 20 cm. negative pressure for 1 min.
3. Release the pressure and place the circular disk on the skin area.

Observe only the central 1 cm. area for petechiae. If less than 4 petechiae appear, the **capillary resistance is normal.** If 4-8 petechiae are present, the test is equivocal and is repeated with 30 cm. negative pressure at another site. More than 8 petechiae should appear at this level of pressure for the test to be considered positive. If more than 8 petechiae appear at −20 cm. pressure in the original test, repetition at −10 cm. at another site simply elaborates on how fragile the capillaries are.

Comments on capillary fragility

Capillary fragility (or capillary resistance) is a reflection of the state of the vascular bed, one of the lines of defense in hemostasis. The vascular tree may be affected by a wide variety of factors which may or may not be related to the clotting mechanism. As a result, abnormalities in capillary fragility must be interpreted as nonspecific phenomena indicating the need for further investigation of the patient.

In the group of capillary purpuras with normal platelet counts, the capillary fragility is variable but at times may be markedly abnormal. We have seen mechanical purpura resulting from application of a tourniquet or blood pressure cuff or associated with wearing of tight garters in patients in whom the capillary fragility test has been generally normal. Griffin[265] has emphasized the abnormal Gothlin indices in a large percentage of patients with diabetes and hypertension. He claims reversal of the abnormality by administration of rutin or other flavanoids. Variable values are likewise noted in sepsis, allergy, drug intoxications, scurvy, uremia, and senility. Ackroyd[260] maintains that in allergy the basic lesion causing purpura is an increased capillary fragility. Other conditions which may be associated with increased capillary fragility without coexisting abnormality of clotting mechanism include hereditary capillary fragility, hereditary capillary telangiectasia, Glanzmann's thrombasthenia, Kaposi's sarcoma, rheumatic heart disease, and leukemia. Abnormal capillary resistance to negative pressure has also been seen in patients with acute myocardial infarction.

Thrombocytopenia is almost universally associated with an increased capillary fragility. This relationship obtains whether the thrombopenia is primary or is secondary to disease, drug intoxication, etc. In such instances the capillary fragility parallels the level of the platelet count and the skin bleeding time. Exceptions to such parallelism are occasionally found. As a rule, the capillary fragility test reflects the severity of the disease and usually returns to normal in patients whose disease improves with treatment, whether the therapy be splenectomy or the removal of secondary factors. Occasionally the capillary fragility may remain abnormal in patients who appear clinically cured by splenectomy.

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*From Quick, A. J.: Hemorrhagic diseases, Philadelphia, 1957, Lea & Febiger.

†Rexall Drug Co., Los Angeles, Calif.

In occasional cases of polycythemia vera, even with a high platelet count, increased capillary fragility is seen, which is also noted in nonthrombocytopenic purpura.

In contrast with thrombocytopenia there are very gross abnormalities of the clotting mechanism which are generally associated with normal capillary fragility. These include hemophilia and hemophilia-like diseases (AHG, PTC, and PTA deficiencies) and fibrinogenopenia.

Hypoprothrombinemia due to any cause may be associated with abnormalities of capillary fragility if the prothrombin level sinks low enough. When the prothrombin status is returned to normal by therapy with vitamin K, the capillary fragility follows the same course.

BLOOD PLATELET COUNTING

There are several technics available for counting blood platelets. In the **indirect** counting method platelets are counted in their relationship to red blood cells on fixed films. This method is least reliable since the results depend on the distribution of platelets and on the red blood cell count. The permanent record is an advantage because it allows study of platelet morphology and gives an impression of the number of platelets.

The **direct** counting method is the most accurate method at the present time. There are 3 methods: (1) Rees-Ecker method, (2) phase microscopy, and (3) Coulter counter (p. 1108).

Indirect counting methods

Method of Dameshek[266]

Reagent:

1. Dissolve 0.15 gm. brilliant cresyl blue in 100 ml. distilled water containing 8 gm. sucrose and 0.4 gm. sodium citrate.
2. Mix well and filter.
3. Add 3 drops 1:10 dilution of formalin (40%) as a preservative.

Procedure:

1. Discard the first drop of blood obtained by puncturing the skin.
2. Place a fairly large drop diluting fluid over the puncture wound and gently press the finger so that a small amount of blood wells up into the drop of staining solution.
3. The proportion of blood to stain should be about 1:5.
4. Transfer the mixture of blood and stain to a cover glass; then invert the cover glass and drop it on a slide.
5. Wait 15-45 min.
6. Examine with an oil immersion objective.
7. Count as directed below.

Reticulocytes can be counted in the same preparation.

Counting blood platelets

Count all the erythrocytes and blood platelets in consecutive fields until 250 erythrocytes have been counted. Count 4 such fields after the 4-field meander method.

Calculate the number of blood platelets in 1 c.mm. blood by the following formula:

$$\frac{\text{Patient's red count}}{1000} \times \text{No. blood platelets counted}$$

$= $ No. blood platelets in 1 c.mm. blood.

Example:

1000 red blood cells counted.
65 blood platelets counted.
Patient's erythrocyte count 5,000,000.

$$\frac{5{,}000{,}000}{1000} \times 65 = 325{,}000 \text{ blood platelets in 1}$$

c.mm. blood.

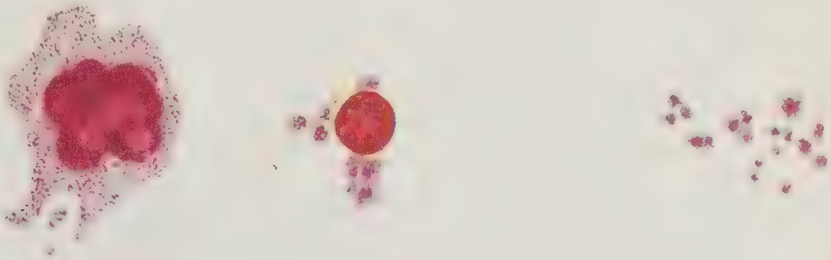

Fig. 399. Development of blood platelets from a megakaryocyte. (Wright stain.)

The **normal** platelet count with the Dameshek method is 500,000-900,000; average 716,000/c.mm.

Direct counting method

Rees-Ecker method

Rees-Ecker diluting fluid[267]:

Sodium citrate	3.8 gm.
Neutral formaldehyde, 40% .	0.2 ml.
Brilliant cresyl blue . . .	0.1 gm.
Distilled water	100 ml.

Filter and centrifuge before use. Keep stoppered in a refrigerator.

Procedure:

1. Draw blood, capillary or venous (aspirated into siliconized syringe and test tube), into a red cell pipet to the 0.5 mark.
2. Draw centrifuged and filtered diluting fluid to the 101 mark.
3. Shake in a pipet rotor for 5-10 min.
4. Expel 4 drops and fill a standard counting chamber, both sides.
5. Allow to stand for 15 min. in a closed Petri dish kept moist with wet filter paper in the top half.
6. Count the platelets in the finely ruled center area of each chamber.

Platelets are bluish and must be distinguished from debris.

Calculation: The counted space measures 1 × 1 × 0.1 c.mm. Since 2 such spaces are counted, the platelets in a space of 0.2 c.mm. are enumerated. To obtain the number of platelets in 1 c.mm., multiply the total count of the 2 chambers by 5 × 200 (dilution factor).

Phase microscopy method of Brecher-Cronkite[268]

Diluting fluid: 1% ammonium oxalate in distilled water is used. Store in a refrigerator and filter before use.

Procedure:

1. Use capillary blood or venous blood obtained by the 2-syringe method; the second syringe should be siliconized.
2. Empty blood into a siliconized test tube.
3. Draw blood to the 0.5 mark in 2 red cell pipets and dilute with diluting fluid to the 101 mark.
4. Shake pipets for 3 min.
5. Expel the first 4 drops and fill both chambers of a hemacytometer. Use No. 1 cover slip.
6. Place the hemacytometer in a closed Petri dish kept humid with moist filter paper in the top half.
7. Count the platelets by phase microscopy in the central sq.mm. of each chamber.

Calculation: This is the same as for the Rees-Ecker method.

The direct counting method should always be supplemented by a blood film to confirm the number counted and to study platelet morphology.

Interpretation: The diagnosis of thrombocytopenia and of thrombocythemia is essentially based on the platelet count. Platelet morphology will help establish the diagnosis of thrombopathia. The platelet count often reflects bone marrow activity. If the bone marrow is depressed, hypoactive, or aplastic, the platelet count will fall. If the bone marrow recovers, the platelet count will improve although it may lag behind several weeks. In certain myeloproliferative syndromes, though the bone marrow may be severely damaged, the platelet count may actually be increased.

CLOT RETRACTION

There are many modifications of the clot retraction test, which at best is only a rough screening test of the clotting mechanism.

1. Obtain 2-3 ml. blood by venipuncture and transfer to a clean test tube, preferably a Wassermann tube.
2. Stopper and leave in a 37° C. water bath.
3. Inspect hourly for beginning clot retraction and then every 2 or 3 hr. for completion of retraction.
4. Observe the quality of the clot and possible lytic activity.

Normal clot retraction: This begins in 1 hr., and a definite margin of serum is seen between the clot and the test tube walls in 2 hr. At the end of 24 hr. the clot contracts fully, leaving an area of clear serum above it and surrounding its sides.

Comments: Strict standardization of this method is necessary since the result will depend on (1) the volume of blood in the test tube, (2) the diameter of the test tube, (3) the temperature of the water bath, (4) the type of glassware, (5) the method of blood collection, (6) the handling of the test tubes, (7) the fibrinogen content of the blood, and (8) the state of the platelets and the plasma coagulation factors present (see discussion that follows).

The test can be rendered more sensitive by siliconizing the test tubes so that the normal range will extend up to 30 min.

Clot retraction depends mainly upon 2 factors: blood platelets and plasma coagulation factors. It was thought at one time that clot retraction depended wholly on the number of platelets. Usually the platelet count is below 100,000/c.mm. in most specimens of blood which do not retract, but occasionally poor retraction is seen when the platelet count is normal, such as in thrombasthenia. In advanced liver diseases with hypoprothrombinemia, clot retraction may be poor. In hemophilia the coagulation time is markedly delayed, but once a clot has formed, it retracts normally. There is some evidence that clot retraction can be correlated to venous thrombosis.

TESTS FOR ABNORMALITIES OF CLOTTING MECHANISM

Determination of clotting time

Capillary tube method

Materials:

1. 3-4 in. capillary tubes.
2. Capillary blood specimen.

Procedure:

1. Obtain a drop of blood by skin puncture and allow to enter several capillary test tubes.
2. Break off small pieces of the tubes at 30 sec. intervals until a fine thread of fibrin persists at the site of separation of fragments of the capillary tube.

The time from skin puncture to the formation of the fibrin thread is the clotting time, measured by a stopwatch.

Normal: 2 to 4 min.

This method is applicable only in infants (venipuncture difficult) and in patients receiving heparin therapy. The unavoidable release of tissue thromboplastin makes this method only a very rough measure of coagulability.

Modified Lee-White method

Materials:

1. 3 clean test tubes.
2. 2 clean syringes, 1 coated with silicone.
3. Water bath.
4. Stopwatch.

Procedure:

1. Draw blood from a vein by a clean puncture, avoiding an undue amount of suction, by the 2-syringe method.
2. Remove the needle from the syringe and gently transfer 1 ml. quantities of the blood specimen to each of the 3 test tubes, prewarmed in a 37° C. water bath.
3. After approximately 5 min., gently tilt the test tube which was filled last (as it contains the potentially contaminated blood drawn first) and observe for clotting. If a clot has not formed, tilt this same tube every 30 sec. until the blood is no longer liquid.
4. At about 1 min. later, check the second tube for clot formation as described for tube 1. Tilt this second tube every 30 sec. until a firm clot is detected and then check the third tube, which is the tube which was filled first (containing the blood least contaminated with thromboplastin).
5. Finally tilt tube 3 until a clot has formed.

The coagulation time is the length of time required for the blood to clot in tube 3.

Normal: 8-14 min.

Clot timers

Two automatic timing devices of clotting are available.

The Clot-Timer* operates on the principle that clotting produces a change in the viscosity and in the surface tension of the clot or plasma. As soon as clotting begins, electrodes automatically stop a counting device.

The Thromboblastograph* makes visual and photokymographic observations simultaneously on 3 different samples of blood.

Thromboblastography is based on the fact that a plunger is inserted into a rotating cap which contains the blood. Attached to the plunger is a mirror which reflects a light beam onto a scale and onto photographic film. As long as the blood is liquid, the plunger will remain stationary even though the cap rotates. As soon as the blood begins to clot, it will carry the plunger with it, and the reflected beam will parallel the mirror movement. This method is useful if a graphic record of hypercoagulability or hypocoagulability of the blood is desired and if fibrinolytic activity or thromboplastin generation are studied.

Plasma recalcification method—Quick[269]

Materials:

1. M/10 Sodium oxalate.
   Prepare by dissolving 1.34 gm. sodium oxalate in 100 ml. distilled water (total).
2. M/100 Calcium chloride.
   Prepare by dissolving 0.11 gm. anhydrous calcium chloride and 0.42 gm. sodium chloride in 100 ml. distilled water.
3. Centrifuge, stopwatch, and venous blood.

Procedure:

1. Draw 4.5 ml. blood by 2-syringe method; remove needle from syringe, and immediately transfer blood to 0.5 ml. oxalate solution.
2. Mix thoroughly by inverting stoppered tube several times.
3. Divide the mixture into 2 approximately equal portions.
4. Centrifuge 1 portion at 1000 r.p.m. for 5 min. (platelet-rich plasma) and the other at 3000 r.p.m. for 5 min. (platelet-poor plasma).
5. Withdraw 0.1 ml. plasma from each specimen and place in separate test tubes in a water bath at 37° C.
6. Add 0.2 ml. M/100 calcium chloride to each sample of plasma and begin timing immediately with a stopwatch.
7. Keep the tubes in a water bath and tilt occasionally until a clot forms. This is the end point of the test. Note the exact time of clot formation.

Normal:

Slow centrifugation: 95-135 sec.

Fast centrifugation: Not more than 15 sec. longer than in slow centrifugation.

Comments: The plasma recalcification time is the time required for a fibrin clot to appear in decalcified plasma. The blood specimen is first prevented from clotting by removing calcium with a known amount of sodium oxalate. Calcium is then added in the form of a known quantity of calcium chloride to start the clotting mechanism.

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*Mechrolaboratories, Inc., Mountain View, Calif.

*Scientific Products Division, American Hospital Supply Corp., Evanston, Ill.

The recalcified plasma clotting time is prolonged in marked hypoprothrombinemia. In deficiencies of antihemophilic globulin (AHG), plasma thromboplastin component (PTC), and plasma thromboplastin antecedent (PTA), the clotting time of recalcified plasma is also prolonged. The degree of prolongation appears to be related to the degree of deficiency. A prominent feature of these deficiencies is that the clotting time of the platelet-poor (rapidly centrifuged) plasma shows a greater prolongation of clotting time than that of the platelet-rich plasma (slow centrifugation).

As a test of the adequacy of plasma thromboplastin, it has been supplanted by the prothrombin consumption test.

Heparin-retarded blood coagulation time of de Takats[270, 271]

Principle: The plasma recalcification time (i.e., the spontaneous clotting of blood) is determined after known quantities of heparin are added to delay the process and increase its sensitivity so that hypercoagulability and hypocoagulability of the blood can be better appreciated.

Materials:

1. Heparin solution.
   Use heparin solution containing 0.004 mg. heparin/0.1 ml. saline, which is made by diluting standard 10 mg./ml. heparin solution 1:250 with isotonic saline.
2. Venous blood freshly drawn, using 2-syringe method.

Procedure:

1. Place 0.1 ml. heparin solution in a clean dry glass test tube in a water bath at 37° C.
2. Add 1 ml. blood to this tube and invert twice.
3. At the end of 12 min., tilt the tube gently at 1 min. intervals until a firm clot has formed. The end point occurs when the tube can be completely inverted without any fluid breaking away from the clot.

Normal: 20-35 min.; average, 27.5 min.

Several modifications of this test have been described. Soulier[272] uses 4 test tubes each containing 0.5 ml. oxalated plasma centrifuged 5 min. at 2000 r.p.m. to which is added M/40 calcium chloride solution which contains increasing quantities of heparin. Heparin concentrations are as follows:

No heparin
0.3 unit/ml.
0.7 unit/ml.
1 unit/ml.

This test can be performed on platelet-rich and platelet-poor plasma to test the antiheparin activity of platelets.

Rosenthal states that the "heparin clotting time appears to be the most valuable single test or index of coagulation." He emphasizes that the test may be used in conditions associated with rapid clotting processes as well as with those having prolongation of coagulation. Prolonged heparin clotting times were found in the deficiency states involving AHG, PTC, and PTA. In these conditions the heparin clotting test was found more sensitive than standard procedures such as the Lee-White technic. In addition to these classic conditions, Rosenthal found that 50% of patients with chronic myelocytic or lymphocytic leukemia had prolongation of the heparin clotting time. This previously unreported coagulation defect was closely related to the degree of hemorrhagic diathesis in these patients even though they did not have thrombocytopenia of a degree usually considered critical.

The heparin clotting time was found prolonged in some patients with bone marrow depression and slight thrombocytopenia, such as may occur in malignant lymphomata and other diseases related to the hematopoietic system. Although these patients showed no clinical manifestations of hemorrhage, they had prolongation of the heparin clotting time.

Rosenthal[273, 274] found evidence of accelerated coagulability in 74% of patients within 3 weeks following the onset of myocardial infarction. The heparin clotting time was shortened in these patients with a mean of 18.7 min., ranging from 11-20 min. Routine clotting times and platelet counts were normal. A control group of patients with cardiovascular disease but without myocardial infarction showed normal values. Dicumarol was found to have marked effect in prolonging the heparin clotting time.

Comments on clotting time determinations

Measurement of the clotting time may or may not disclose the presence of an abnormality in the clotting mechanism. Finding a prolonged clotting time should always initiate further investigation of disturbances in coagulation. Since prolongation of the clotting time may be due to deficiencies of plasma factors, such as AHG, PTC, PTA, fibrinogen, accelerator factors, and prothrombin, special procedures directed at determining these specific defects must be pursued. Anticoagulants, either acquired or idiopathic, may have to be considered. A screening procedure for determining whether there is a deficiency or an anticoagulant has been described by several authors,[261, 275-280] using technics of matching mixtures of blood.

Differentiation of plasma defects and circulating anticoagulants

Principle: When an abnormal clotting time has been found, the question arises as to whether it is due to a plasma defect or to a circulating anticoagulant. Differentiation of these two mechanisms can be made by determining the coagulation time of mixtures of normal and abnormal blood.

Materials: The same materials as in the plasma recalcification method of Quick (p. 1199) are used.

Procedure (adapted to method of Cartwright[281]):

1. Add 4.5 ml. venous blood from the patient to 0.5 ml. M/10 sodium oxalate.
2. Repeat this procedure in another tube, using blood of a normal subject.
3. Mix each sample thoroughly.
4. Centrifuge both specimens of oxalated blood in the same centrifuge at 1000 r.p.m. for 5 min.
5. Prepare a series of mixtures of the 2 plasmas as follows:

	Tube					
	1	*2*	*3*	*4*	*5*	*6*
Patient plasma (ml.)	0.2	0.4	0.1	0.1	0.1	0.0
Normal plasma (ml.)	0.0	0.1	0.1	0.2	0.4	0.2

6. Mix the contents of each tube thoroughly.
7. Transfer 0.1 ml. samples of each tube to 6 clean test tubes and place in a water bath at 37° C.
8. Add 0.2 ml. M/100 calcium chloride to each tube, mix, and begin timing immediately with a stopwatch.
9. Observe each tube for firm clot formation and record time.

Tube 6 is the **normal control** and should have a clotting time of 95-135 sec.

Tube 1 should have a clotting time prolonged beyond 135 sec.

If the patient's condition is due to a deficiency in some plasma factor necessary for coagulation, the addition of oxalated normal plasma will correct the deficiency, and under such conditions tubes 2-5 will have normal coagulation times.

If the disorder in the patient is due to a circulating anticoagulant, tubes 2-5 will show increased coagulation times, with progressively diminishing values from tubes 2-5.

After it is ascertained whether the coagulation defect is due to deficiency of plasma factors or to circulating anticoagulant, special tests must be carried out to ascertain the specific elements involved. These include the following procedures:

A. Tests for plasma deficiencies
  1. Partial thromboplastin time (PTT)[282]
  2. Assay of antihemophilic factor[282]
  3. Prothrombin time
  4. Prothrombin consumption time (plasma or serum)
  5. Studies of mixtures
  6. Fibrinogen determination
  7. Thromboplastinogen activity test[283]
  8. Thromboplastin generation test[284]

B. Tests for anticoagulants
  1. Antithrombin and antithromboplastin[285]
  2. Hyperheparinemia[286]

TESTS FOR PLASMA DEFICIENCIES

Prothrombin time determinations

Prothrombin time is the clotting time obtained when an excess of thromboplastin and calcium is added to oxalated plasma under standardized conditions.

Plasma prothrombin time—Quick[287] one-stage prothrombin time

Reagents:

1. M/10 Sodium oxalate.
   (a) Dissolve 1.34 gm. sodium oxalate in enough distilled water to make 100 ml.
2. M/40 Calcium chloride.
   (a) Dissolve 0.28 gm. anhydrous calcium chloride in enough distilled water to make 100 ml.
3. Thromboplastin solution.

Use commercially available rabbit or human brain thromboplastin preparation (Simplastin*) to which calcium chloride and sodium chloride have already been added.

Prepare thromboplastin solution exactly according to the manufacturer's instructions.

Procedure:

1. Into a conical graduated centrifuge tube measure 0.5 ml. M/10 sodium oxalate.
2. Withdraw venous blood with special precautions to avoid trauma and add exactly 4.5 ml. to the oxalate and mix quickly to prevent clotting.
3. Centrifuge at 1500 r.p.m. for 5 min.
4. Withdraw plasma with a dropper and place in a test tube; then transfer the tube to a 37° C. water bath.
5. Pipet 0.2 ml. reconstituted Simplastin into at least 4 test tubes and then place them in the 37° C. water bath for at least 3 min.
6. With a 0.1 ml. micropipette transfer 0.1 ml. whole plasma previously warmed to 37° C. to the tube containing the warmed 0.2 ml. Simplastin.

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*Warner-Chilcott Laboratories, Morris Plains, N. J.

7. Blow the plasma out of the pipet quickly and start the timer simultaneously.
8. Keep the tube warmed and tilt about twice per sec.
9. When the first evidence of clotting appears, stop the stopwatch and record the time.
10. Repeat the test with a normal control, e.g., Protrol.*

Report prothrombin time in sec. or as **% normal activity,** always recording the control results as well.

If the venous blood to be tested is kept at room temperature, the prothrombin time should be determined within 1 hr. after collection. If the venous blood is kept at 4° C., the prothrombin time should be determined within 4 hr.

With an active preparation of thromboplastin, **normal human plasma** will clot consistently in 12-14 sec.

Comments on Quick one-stage prothrombin time

Since Quick's original publication, numerous modifications of the method have been devised in order to simplify the procedure for routine clinical laboratory use, improve on the reagents, or correct certain objections to the basic principles in the test.

The activity of a given batch of thromboplastin should be checked against normal controls. To circumvent the possibility that the so-called normals are not truly normal, commercial preparations of lyophilized plasma are available as controls.[288]† The prothrombin time of an unknown sample is greatly dependent on the thromboplastin potency and should be reported in seconds, with an accompanying note of the time for the normal control.

Curves are constructed with varying dilutions of plasma, with time (sec.) as one component and percentages as another. Such calibration curves are used as references for reporting tests in percentages. An objection to the method as originally reported by Quick is that in the higher percentage ranges the slope of the curve is so steep that small increments of time reflect large changes in percentages. To circumvent this objection, various dilutions of the test plasma have been suggested. Usually the dilution is in the range of 1:10 but may be adjusted at other levels for special situations. Although saline solution has been recommended as the diluent by some, it is now generally agreed that it should not be used because it dilutes all coagulation factors in plasma. Prothrombin-free plasma is one of the diluents most universaly recommended. There is some question as to whether this should be human or animal plasma. The adsorbents used for preparation of such prothrombin-free plasma have been barium sulfate or passage through filter paper pads containing high concentrations of asbestos.

A method that considers many of these factors is the one by Rosenfield and Tuft[289] as modified by Alexander and deVries[290] and is recommended.

Prothrombin time—Rosenfield-Tuft,[289] modified by Alexander-deVries[290]

Reagents:

1. M/10 Sodium oxalate.
   (a) Dissolve 1.34 gm. anhydrous sodium oxalate in 100 ml. distilled water.
2. M/10 Potassium oxalate.
   (a) Dissolve 8.423 gm. crystalline potassium oxalate in distilled water and dilute to 1000 ml. with distilled water.
   (b) Use 1 ml. this solution as anticoagulant for 9 ml. blood, which is used for the preparation of prothrombin-free plasma.
3. M/40 Calcium chloride.
   (a) Dissolve 2.77 gm. purified anhydrous calcium chloride in distilled water and dilute to 1000 ml. with distilled water.
4. Thromboplastin: See Quick method and comments (p. 1201).
5. Prothrombin-free human plasma:
   (a) Collect 9 ml. blood from each of at least 5 normal subjects and place each in 1 ml. M/10 potassium oxalate and mix.
   (b) Separate the plasma and pool.
   (c) Divide the plasma pool into 2 portions, using one for adsorption with barium sulfate and the other for diluting the normal or test plasma.
   (d) Add 100 mg. barium sulfate powder for each ml. plasma, shake thoroughly, and incubate for 10 min. at 37° C.
   (e) Centrifuge at 3000 r.p.m. for 30 min. and pour off the supernatant fluid.

Refrigerate this at 4° C. until ready for use. Since this material must be used within 2 hr. after its preparation, Frommeyer[291] recommends lyophilization in order to maintain its potency.

Lyophilized prothrombin-free plasma is reconstituted with buffer at the time of its use.

Preparation of curve for time/percentage relationships: Dilute **fresh, pooled, normal** plasma serially (10, 12½, 20, and 30%) with the prothrombin-free plasma prepared as just described. On each dilution perform a Quick prothrombin time test (p. 1201) and plot a curve with the time in seconds against the concentration in per cent (Fig. 400).

Procedure:

1. Place 0.5 ml. M/10 sodium citrate in a test tube.
2. Add 4.5 ml. venous blood and mix.

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*Knickerbocker Laboratories, New York, N. Y.

†Diagnostic Plasma, Warner-Chilcott Laboratories, Morris Plains, N. J.

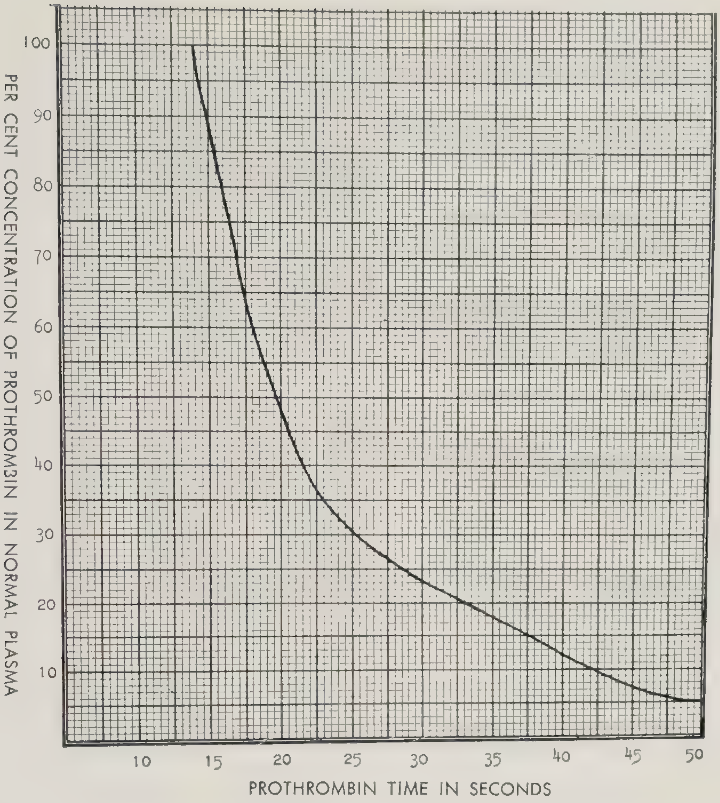

Fig. 400. Curve constructed for Quick prothrombin time determination. Such calibration curves are used as references in reporting prothrombin time in percentage.

3. Centrifuge at 1000 r.p.m. for 5 min.
4. Remove the supernatant plasma with a clean pipet and transfer it to a clean test tube.
5. Place all the reagents and tubes used hereafter in a water bath at 37° C.
6. Place 0.1 ml. plasma and thromboplastin solution in a clean Kahn tube and shake gently to ensure mixing.
7. With stopwatch in hand, blow in 0.1 ml. of M/40 calcium chloride solution, and begin timing.
8. Remove the tube from the water bath periodically and observe the time required for a clot to form; check the concentration in percentage on the curve prepared as shown in Fig. 400.

Comments: The first steps in prothrombin time determination with this test are essentially the Quick technic, with a time-concentration curve prepared by dilution with prothrombin-free plasma. If the result of this determination falls in the flat part of the time-concentration curve, 25 sec. or longer, the result may be recorded as a final figure. However if a shorter time is consumed, it is recommended that the unknown plasma sample be diluted 1:10 with the prothrombin-free plasma, **not saline solution,** and the prothrombin time on a 0.1 ml. aliquot of this be determined as in the method for undiluted plasma. The result in per cent concentration is then multiplied by 10.

Clot-timer technic

Reference has been made to automatic clot-timers,* which are well suited for the determination of the 1-stage prothrombin time. The plasma is placed in a flat metal cuvet which is kept at 37° C. A rotor rotates through the mixture of thromboplastin, calcium chloride, and plasma and stops as soon as a fibrin band produces contact between it and a nonmovable rod.

Discussion of one-stage prothrombin time method

The speed of the formation of the clot depends on 4 clotting factors: prothrombin, factor V, factor VII, and Stuart-Prower factor, assuming that fibrinogen and circulating anticoagulant activity are normal. Because of the fact that this test embraces 4 factors, it is thus possible that a reduction of prothrombin activity of 40-50% of normal, as may occur in acute hepatitis, may be completely masked by factor V and factor VII activity. Similarly, a reduction of factor VII up to 30% will not affect the 1-stage prothrombin time, whereas a mild degree of reduction of both prothrombin and factor VII will by synergistic action prolong the prothrombin time. The concentration of factor V appears to be critical and is not influenced by vitamin K_1 (production of prothrombin and of factor VII is dependent on vitamin K_1). Factor VII is of importance only if reduced.

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*Mechrolaboratories, Mountain View, Calif.

It has long been known that clotting defects accompany liver disease.[293] These defects vary according to the underlying liver disease. In acute viral hepatitis, PTA, Stuart-Prower, and PTC are most markedly reduced; next follow factor VII and prothrombin; least affected is factor V. In a few cases, factor V, factor VII, and prothrombin are actually increased.

In chronic hepatitis and cirrhosis the factors may be within normal limits, but factor VII is frequently decreased, and then may follow factor V and prothrombin as well as the antithrombins.

The 1-stage method is the most common method for controlling anticoagulant therapy with coumarin and related drugs. These drugs reduce both prothrombin and factor VII activity. Initially, within the first 24 hr., factor VII is more rapidly depressed and within a few days there follow prothrombin and Stuart-Prower factor. After administration of vitamin K_1, factor VII recovers more rapidly than does prothrombin. From the preceding discussion it is clear that a prolonged prothrombin time may be due to variations in 3-4 factors or to changes in only 1 or 2 of them.

Since vitamin K_1 administration will correct prothrombin and factor VII deficiencies, provided that liver function is adequate, but will not correct factor V deficiency, which is also not corrected by transfusion of stored blood, it is at times advisable to utilize correction studies to determine which factor is involved in the prolongation of the prothrombin time. These correction studies are not quantitative studies, but they allow separation of factor V, factor VII, and prothrombin deficiencies.

Correction studies of one-stage prothrombin time

Prepare the following mixtures and perform 1-stage prothrombin time tests as follows:

0.9 ml. patient's plasma + 0.1 ml. stored serum (supplies factor VII).

0.9 ml. patient's plasma + 0.1 ml. fresh $BaSO_4$ plasma (supplies factor V).

0.9 ml. patient's plasma + 0.1 ml. stored oxalate plasma (supplies prothrombin).

Whichever of the added factors corrects the originally prolonged prothrombin time can be considered the factor primarily responsible for the prolongation.

Table 241. *Main coagulation factors in various blood fractions used in correction studies**

	Normal plasma	*Normal serum*	*Aluminum hydroxide, $Al(OH)_3$, barium sulfate, $BaSO_4$, adsorbed plasma*
Fibrinogen	+	−	−
Prothrombin	+	−	+
Factors V and VI	+	−	−
Factor VII	+	+	+
Factor VIII	+	−	−
Factor IX	+	+	+
Factor X	+	+	−

*From Bauer, J. D., Toro, G., and Ackermann, F. G.: Bray's clinical laboratory methods, St. Louis, 1962, The C. V. Mosby Co.

Microprothrombin method

Miale[292] described a micromethod for prothrombin determination of oxalated blood which is based on the 1-stage prothrombin method of Quick and is useful if patients resent frequent venipunctures.

One-stage prothrombin method—bedside technic (Manchester technic)

This procedure is indicated if venipuncture is impossible, but it cannot be correlated with the Quick 1-stage method.

One drop of blood from a skin puncture is placed on a glass slide and mixed with one drop of thromboplastin. The periphery of the mixed drop is touched at intervals of 2-3 sec. with a glass rod until fibrin formation is noted. Illumination by transmitted light aids the visualization of the fibrin threads. A normal control should be run with each test. It will clot in 15-25 sec.

Two-stage method of prothrombin time—Warner, Brinkhous, and Smith[294-296]

The basic principle of this method is the measurement of thrombin yield against a standardized solution of fibrinogen. Just as with other prothrombin time methods, the 2-stage technic has undergone numerous modifications. Its complexity has been a bar to routine technical use, but it has served as a useful investigative tool.

The prothrombin methods to be described are the result of an increasing awareness that the Quick 1-stage prothrombin time has certain defects. Thus it may happen that a patient whose prothrombin time is within the therapeutic range may hemorrhage. On the other hand, patients who are apparently well controlled on anticoagulant therapy may develop thrombotic episodes. The previously mentioned dilution of the plasma represents an attempt to increase the sensitivity of the 1-stage method. It is also believed that changes in fibrinogen and factor V concentration might influence the prothrombin time. The P and P method of Owren and Aas was originally introduced to reflect changes of prothrombin and proconvertin (factor VII) only; hence the name P and P.

P and P method of Owren and Aas[297]

The theoretical basis of the test is as follows. The amount of thrombin formed by the interaction of prothrombin and thromboplastin is a measurement of the concentration of prothrombin, as it is assumed that 1 unit of thrombin will be produced by 1 unit of prothrombin. Thus the amount of thrombin produced can be taken as a measure of the amount of prothrombin present in the plasma. In this method, plasma is defibrinated and then diluted with oxalated saline solution. This diluted plasma is then added to a mixture of thromboplastin, calcium, and buffer. The thrombin generated is measured by adding a fixed amount of this mixture to a standard solution of fibrinogen.

The disadvantages of this method lie in the action of plasma antithrombins and the fact that concentration of factor V and factor VII prevents a stoichiometrical conversion of prothrombin to thrombin.

Reagents:

1. Thromboplastin derived from human brain.
   - (a) Free human brain from pia and blood vessels.
   - (b) Wash and grind the gray substance in a mortar with 0.9% sodium chloride solution to a fine emulsion. Emulsification is facilitated by applying a mechanical blender.
   - (c) Dilute the emulsion with 0.9% saline to which 20% Veronal buffer is added. Approximately 5 ml. solution should be used for each gm. brain substance.
   - (d) Allow the extract to stand for about 2 hr. at 45° C.
   - (e) Strongly centrifuge for 15 min.
   - (f) Discard the precipitate.
   - (g) Freeze the grayish colloidal suspension at −20° C. in portions corresponding to the daily requirement. Before using, thaw it at 37° C.

   This extract gives a Quick prothrombin time in normal plasma of approximately 15 sec. When stored frozen at a temperature below − 20° C., the extract remains unchanged during several months.

   NOTE: There are a number of good thromboplastin preparations on the market which can be substituted for this human brain thromboplastin.
2. Veronal buffer, pH 7.35.
   - (a) To 570 ml. 0.1M sodium diethylbarbiturate add 430 ml. N/10 hydrochloric acid and 5.67 gm. sodium chloride.
   - (b) Dilute with an equal volume of 0.9% sodium chloride solution.
3. Diluting solution.
   - (a) To 800 ml. 0.9% sodium chloride solution add 200 ml. Veronal buffer pH 7.35 and 700 mg. potassium oxalate monohydrate.[298]
4. Calcium chloride solution. This is usually 0.03M, but optimal concentration must be determined for each batch of ox plasma.
5. Prothrombin-free and proconvertin-free ox plasma.
   - (a) Mix 9 vol. ox blood with 1 vol. 2.5% (w/v) potassium oxalate monohydrate solution and obtain plasma by centrifugation.
   - (b) Remove prothrombin by using asbestos paper filter pads containing 30% asbestos.
   - (c) Store plasma overnight at 0° C.
   - (d) Centrifuge to remove fatty material which tends to clog filters.
   - (e) Pass the plasma through a clarifying filter containing 20% asbestos and then once through filter paper pads containing 50% asbestos; 400 ml. plasma may be passed through a filter of 15 cm. diameter. When larger amounts are filtered, some prothrombin remains in the filtrate.
   - (f) Adjust the reaction of the plasma to pH 7.3 by adding N/2 hydrochloric acid.
   - (g) Store at −20° C. or lower temperatures in small bottles or glass tubes stoppered with corks in quantities suitable for 1 day's use.
6. Potassium oxalate solution, 2%.
   - (a) Dissolve 2 gm. potassium oxalate in distilled water and dilute to 100 ml. with distilled water.

Procedure:

1. Place 0.5 ml. 2% potassium oxalate in a test tube.
2. Add 4.5 ml. blood obtained by venipuncture and mix.
3. Centrifuge for 5 min. at 1000 r.p.m. and remove the plasma.
4. To 0.2 ml. plasma add 1.8 ml. diluting solution.
5. Place all the reagents in a water bath at 37° C.
6. Place 0.2 ml. prothrombin-free, convertin-free ox plasma in a small Kahn tube.
7. Add 0.2 ml. plasma dilution and 0.2 ml. thromboplastin.

8. Mix by shaking gently.
9. With a stop watch ready, blow out 0.2 ml. calcium chloride solution and begin timing.
10. Observe the end point for clotting.

Record the time as in Quick 1-stage method. Obtain the percentage from a correlation graph. Prepare the graph by serial dilutions of normal plasma, using diluting fluid, but maintaining a concentration of 100 mg.% oxalate.[298]

Comment: Owren believes that by following his procedure he maintains the following principles:

1. Determines combined effect of prothrombin and proconvertin.
2. Keeps a constant substrate of thromboplastin, calcium, proaccelerin, and fibrinogen, the latter 2 coming from the added adsorbed ox plasma.

He particularly points out that his P and P method is superior to previous methods in the control of Dicumarol therapy, in which the effect is a depression of the prothrombin and proconvertin values, leaving proaccelerin unaltered. In vitamin K deficiency and in liver disease, the same relationships hold although in the latter severe damage may cause a decrease in proaccelerin as well.

Specific determination of prothrombin—Owren[299]

The basic principle in this test is to keep the reaction mixture constant in the content of thromboplastin, calcium, proconvertin, proaccelerin, and fibrinogen.

Reagents: Reagents are the same as in the P and P method just described, with the addition of proconvertin solution.

1. Proconvertin.
   Proconvertin is obtained from human serum by adding a small amount (3 ml./100 ml. blood) of thromoplastin to venous blood, stirring immediately after withdrawal. This results in disappearance of prothrombin within 10-30 min. Storage at room temperature for 2 days causes accelerin to disappear.
2. Plasma-serum reagent.
   Mix the serum prepared as just given with equal parts of prothrombin-free and proconvertin-free ox plasma to make a plasma-serum reagent.

Procedure: The method is essentially that of the P and P method, with the following quantities used in this test:

0.2 ml. 1:10 dilution of test plasma.
0.4 ml. plasma-serum reagent.
0.2 ml. thromboplastin.
0.2 ml. calcium chloride.

Record the time required for clotting in this mixture. Calculate the percentage prothrombin by reference to a correlation graph prepared in a manner similar to that in the P and P method.

Comments: The major point about this modification is that it actually measures the activity of **prothrombin only,** whereas the usual one-stage modified Quick technics and the P and P method of Owren measure both prothrombin and proconvertin. In following patients with Dicumarol therapy, the P and P method is desirable because both prothrombin and proconvertin are affected. The determination of prothrombin only is a helpful tool in research.

Specific determination of proconvertin—Owren

The basic principle involved in this test is to keep the reaction mixture constant in thromboplastin, calcium, proaccelerin, prothrombin, and fibrinogen.

Reagents: The thromboplastin and calcium solutions are the same as in the P and P test (p. 1205).

Proconvertin-free ox plasma is prepared by filtering oxalated ox plasma through an asbestos filter pad containing only 20% asbestos.

Procedure: The procedure is essentially that of the P and P method, with the following quantities being used:

0.2 ml. proconvertin-free ox plasma.
0.2 ml. thromboplastin.
0.2 ml. unknown plasma diluted 1:20.
0.2 ml. calcium chloride.

Record the time required for clotting. Calculate the percentage proconvertin by reference to correlation graphs prepared from normal plasma diluted 1:20.

Comments: This determination has been a helpful tool in the investigation of clotting mechanisms. In clinical studies of bleeding diatheses, Alexander et al.[300] and Owren[302] and Aas[301] have described congenital hypoproconvertinemia. In 1953 Owren[302] described this defect as a congenital and hereditary defect. He points out that the whole clotting time in these patients may be normal or only slightly prolonged. The Quick prothrombin time was markedly prolonged, but vitamin K did not restore the hypoproconvertinemia to normal.

Specific determination of proaccelerin—Owren[301]

The basic principle involved in this test is to keep a reaction mixture constant in thromboplastin, calcium, prothrombin, proconvertin, and fibrinogen.

Proaccelerin-free plasma for the test is obtained by storage of adsorbed ox plasma or from a case of parahemophilia.

Procedure: The procedure is the same as in the P and P method (p. 1205), using the same concentrations of calcium chloride and thromboplastin with proaccelerin-free plasma.

Comments: This method has been helpful as an investigative tool in the study of clotting mechanisms. By this method, Owren in 1943 was able to detect the first verified case of parahemophilia (factor V deficiency) as a human congenital deficiency. Since then, other cases have been reported as either hereditary or congenital.

These clinical studies have given a new impetus to the study of hemophilia and hemophilia-like diseases.

Prothrombin consumption method—Quick[303]

Reagents:

1. Blood.
   - (a) Obtain 6 ml. blood by clean venipuncture, using 2-syringe silicone technic.
   - (b) Transfer 2 ml. samples to each of 3 tubes.
2. Deprothrombinized rabbit plasma.
   - (a) Mix 9 vol. freshly drawn rabbit blood with 1 vol. M/10 sodium oxalate.
   - (b) Obtain the plasma by centrifugation.
   - (c) Measure the plasma after withdrawing it from the blood cells.
   - (d) For each ml. oxalated plasma, place 2.5 ml. 0.005M suspension of tricalcium phosphate in a test tube.
   - (e) Centrifuge to pack the gelatinous calcium phosphate.
   - (f) Pour off the surplus water.
   - (g) Add the required vol. fresh oxalated plasma.
   - (h) Mix with the adsorbent.
   - (i) Stir repeatedly with a small glass rod for 5 min. at room temperature.
   - (j) Remove the calcium phosphate by centrifugation, pouring the clear adsorbed plasma into a clean test tube.
3. Thromboplastin solution.
   - (a) Incubate 0.2 gm. acetone-dehydrated rabbit brain with 5 ml. physiologic saline at 50° C. for 20 min.
4. Tricalcium phosphate, 0.005M.
   - (a) Dissolve 158 gm. trisodium phosphate in 1000 ml. distilled water.
   - (b) Dissolve 66.6 gm. anhydrous calcium chloride in 1000 ml. distilled water.
   - (c) Pour the calcium chloride solution into the trisodium phosphate solution with thorough stirring.
   - (d) Adjust the reaction to pH 7.0.
   - (e) Wash the precipitated tricalcium phosphate repeatedly by decantation until the sodium chloride is removed.
   - (f) Dilute to 1 L., thus making a 0.2M suspension.
   - (g) From this stock, a 0.005M solution can be prepared by mixing 2.5 ml. with 97.5 ml. distilled water.
5. 0.02M Calcium chloride.

Procedure:

1. Place the 2 ml. samples blood in a water bath at 37° C.
2. 15 minutes after clotting has occurred, remove tube 1, centrifuge at top speed for 1 min., and determine the serum prothrombin time by adding 0.1 ml. serum to a mixture of 0.1 ml. deprothrombinized rabbit plasma, 0.1 ml. thromboplastin, and 0.1 ml. calcium chloride solution.
3. Blow in the serum, and time.
4. Observe for clot formation and record time.
5. At the end of 30 min., centrifuge the second tube and perform a prothrombin time on this serum in the same manner as in the first tube.
6. Repeat the prothrombin time on the first tube at this time.
7. At the end of 45 min. recheck the prothrombin times of the sera in tubes 1 and 2.
8. At the end of 1 hr. centrifuge the third tube and make prothrombin time determinations on the sera of all 3 tubes.

Normal: 30-150 sec.

Comments: See comments on the simplified serum prothrombin test for the basic principles involved.

In this test the serum prothrombin time of normal blood ranges from 30-150 sec. The 3 tubes tend to show increasing times from the 15 min. test to the 1 hr. test. The testing of 3 tubes in the manner recommended serves as a control over the technic.

In hemophilia the times vary between 10 and 20 sec., with very little change in all 3 tubes. In thrombocytopenic purpura and thrombasthenia the average time is 25 sec. and in parahemophilia it is 34-38 sec.

Simplified serum prothrombin consumption test[304]

Materials:

1. Serum.

   Serum obtained from venous blood, using 2-syringe silicone technic and allowed to clot at room temperature. Place the blood in a water bath at 37° C. for 1 hr. after clotting. Centrifuge for 3 min. to separate the serum. Test the serum immediately or store at 4° C. for 1 hr. Do not use if stored more than 1 hr.
2. Thromboplastin solution.*

   Prepare as for plasma prothrombin time.
3. Fibrinogen solution.†

   This is to contain 300 mg.% fibrinogen in 0.85% sodium chloride solution.

For use: Just before the test, mix 2 ml. thromboplastin solution with 1 ml. fibrinogen.

Procedure:

1. Place all reagents in a water bath for 5 min.
2. Place 0.2 ml. fibrinogen-thromboplastin mixture in a clean dry test tube.
3. Add 0.1 ml. test serum to mixture by blowing it out of the pipet, simultaneously starting the timer.
4. Observe for clot formation, and time.

Normal: Longer than 30 sec.

Comments: The basic principle of this test and of the Quick prothrombin consumption test is that, in the normal process of clotting of blood, prothrombin is almost completely consumed, leaving serum with little residual prothrombin. If there is a deficiency of thromboplastin complex in a blood, prothrombin consumption is diminished, leaving a high prothrombin residual in the serum.

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*Simplastin; Warner-Chilcott Laboratories, Morris Plains, N. J.

†Warner-Chilcott Laboratories, Morris Plains, N. J.

If to these sera are added optimal amounts of fibrinogen and thromboplastin, the time for a clot to form reflects the amounts of prothrombin present. In normal blood, the time is prolonged (beyond 30 sec.). A time of 20-30 sec. is considered doubtful, and anything below 20 sec. is indicative of thromboplastin deficiency.

In thrombocytopenia, whether primary or secondary, the test has yielded low values. This has been true of a case of thrombasthenia. Hemophilia and hemophilia-like diseases give a short serum prothrombin time. When heparin is administered, the serum prothrombin time becomes abnormally low. On the other hand, the coumarins have no effect on the serum prothrombin time since they do not depress thromboplastin complex.

Sources of error in prothrombin time estimations

1. The venipuncture must be clean.
2. The ratio between oxalate and blood must not be changed. Excess of oxalate will materially alter the results.
3. In centrifuging the blood, the correct time and speed are 5 min. at 2000 r.p.m.
4. Plasma should be removed from the cells as rapidly as possible after centrifuging.
5. Plasma must be kept refrigerated if not handled immediately.
6. The temperature of the water bath must be **exact.**
7. Plasma standing at 37° C. in a water bath loses 50% of its activity within 1 hr.
8. Any detergent used in cleaning glassware must be removed; if left on the glassware, even if only a very faint trace remains, it will interfere with the accuracy of the test.

Thrombotest[305-309]—Owren

After the introduction of the Owren P and P test it was discovered that the anticoagulants reduce not only prothrombin and factor VII but also factor IX. In an attempt to produce a sensitive test which would allow the clinician to follow a patient on anticoagulant therapy, Owren prepared an all-in-one reagent called thrombotest.

Thrombotest contains phospholipids (cephalin) from human brain or soya bean, calcium chloride, crude extracts of animal tissue (thromboplastin), and adsorbed bovine plasma from which factors VII, IX, and X and prothrombin have been removed. Factor V and fibrinogen remain. The test can be made on venous or capillary blood. At the present time, thrombotest reagents are available from only one manufacturer.* The reagent, already mixed, is supplied in lyophilized form and is reconstituted with distilled water for testing of capillary blood and with 3.2 mM calcium chloride for testing citrated blood. The result of this rapid and simple test is expressed as a percentage of normal read from a correlation curve prepared from dilutions of pooled normal plasma. **Normal blood clotting time** is 35-40 sec.

Comments on prothrombin time determinations

The determination of prothrombin time has become so much a part of daily clinical practice that it is now a household word in the physician's practice. The background development of this interest is typical of how investigations and investigators, great distances apart, are suddenly catalyzed. In fact, a greatly stimulated interest in all phases of coagulation can be said to begin with the epic discovery by Dam,[310] in 1929, that chicks developed a hemorrhagic syndrome on a synthetic diet.[311] By 1935, Dam[312] reached the conclusion that these chicks were suffering from a vitamin deficiency with a coagulation defect (Koagulation-vitamin or vitamin K). The calendar of events that followed are listed below:

1936: Chicks on artificial diet have prothrombin deficiency.[313] Quick's quantitative studies on chicks showed prothrombin deficiency; hemorrhage occurred at levels of 10% prothrombin; alfalfa restored chicks to normal.[314] Quick suggests hypoprothrombinemia of patients with obstructive jaundice is related to vitamin K deficiency.

1937: Quick[315] reports hypoprothrombinemia and hemorrhagic diathesis in patient with obstructive jaundice. Tried treatment with alfalfa but unsuccessful. Suggested combining bile salts with alfalfa.

1938-1939: Clinical application of Quick's suggestions.[316-318]

1939: Isolation of vitamin K by Doisy and his associates.[319-322]

1939-1940: Chemical structure of vitamin K[319-321, 323, 324] and its synthesis; vitamin K_a naphthaquinone.

1940: The race is on for therapeutic use of synthetic vitamin K.[325-328]

Many reviews of the story are available, and we heartily recommend Quick's review on the hemorrhagic diathesis of avitaminosis K[285] as exciting reading. To surgeons everywhere there came a very simple method of controlling distressing oozing of blood in the patient with obstructive jaundice. The administration of vitamin K to such patients has become so routine as to warrant little further comment. It was quite natural that other clinical states associated with a deficiency of vitamin K be sought; perhaps the other outstanding group is the hemorrhagic disease of newborn infants. Prophylactic treatment of the mother just prior to delivery has brought about the same striking results as with the jaundiced patient. It has become routine procedure in obstetrics to administer vitamin K during labor. Lesser in numbers involved are hypoprothrombinemic states associated with vitamin K deficiency due to the following disorders: (1) biliary fistula, (2) poor absorption from the gastrointestinal tract, as in ulcerative colitis, sprue, intestinal fistulae, intestinal obstruction, pellagra, and gastrocolic fistula, (3) artificial fever, (4) change of the intestinal flora by sulfonamides and antibiotics, and (5) liver disease.

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*Nyegaard & Co., Oslo, Norway.

Just as prothrombin determinations may disclose a vitamin K deficiency, serial determinations are helpful in judging the efficacy of vitamin K therapy. If a given clinical state is due to avitaminosis K, administration of the vitamin parenterally or orally should result in a fairly prompt return of prothrombin to normal **provided that the liver function is normal.** This fact forms the basis of a liver function test in which standardized doses of vitamin K are administered and prothrombin time determinations are made subsequently.[285]

If a patient has hypoprothrombinemia which promptly responds to vitamin K therapy, he does **not** have severe liver damage. The corollary to this is that a poor response indicates either parenchymal liver disease or inadequate dosage.

An interesting situation has been reported in which patients apparently failed to respond to vitamin K therapy, and the condition was called idiopathic hypoprothrombinemia until all the facts became known. In all these patients self-medication with Dicumarol should be suspected.

As the saga of vitamin K deficiency was exploding, it was not at all surprising that Link and associates, with the guidance of Quick, promptly solved a mystery plaguing farmers since 1921. Cattle developed a hemorrhagic disease of serious economic proportions which attracted the attention of veterinarians and research men. The events which followed are listed below:

1922-1924: Schofield[329, 330] attributes disease to spoiled clover ingested by cattle and associated with abnormal blood clotting.

1937: Quick[314] demonstrates drop in prothrombin of cattle fed toxic sweet clover hay.

1941: Link and associates[331-333] isolate, identify, and synthesize active agents in sweet clover, 3,3′-methylenebis (4-hydroxycoumarin).

1941: Studies on effect of dicoumarin on human beings and experimental animals[334, 335]; the beginning of Dicumarol therapy.

Since these basic studies, anticoagulant therapy has been extensively used in the treatment of human disease associated with thrombosis.

Anticoagulant therapy

The patient, the laboratory technician, the pathologist, and the clinician should be familiar with the difficulties which may arise in the course of anticoagulant therapy. The clinician must know the interpretation and limitations of the various tests used to follow the course of the patient. The clinical pathologist and the laboratory technician should be familiar with the various anticoagulants—their indications, contraindications, control tests, and antidotes. Last, the patient should know the danger signs of uncontrolled self-medication and of unsupervised discontinuation of anticoagulant therapy.

Dicumarol

Two substances frequently used as clinical anticoagulants are Dicumarol (a coumarin derivative) and heparin.

Dicumarol (bishydroxycoumarin) (3,3′-methylenebis-4-hydroxycoumarin) has been well established as an adjunct in the management of thromboembolic disease. Perhaps its most universal application in clinical medicine has been in acute coronary thrombosis. Coronary occlusion has been known to be associated with the formation of mural thrombi which may serve as a nidus for emboli. Prophylactic administration of Dicumarol reduces the incidence of emboli considerably.

Wright et al.[336] come to the conclusion that all patients with myocardial infarction should receive anticoagulant therapy unless a contraindication to such therapy exists.

Schnur,[337] in a question-answer address read in a panel discussion on the use and abuse of anticoagulant therapy, pointed out that not all of the problems relating to such therapy are readily solved. He particularly indicated the difficulties of the clinical diagnosis of thromboembolism and even questioned whether postmortem examinations can fully reflect this feature of mural thrombi. He believes that the effective ranges of prothrombin at present are at best "educated guesses." His position is that of selective therapy rather than routine. This subject is pursued further by Russek and Zohman[338] who came to the following conclusion: "Anticoagulant therapy is neither necessary nor desirable for the large segment of patients who sustain their first attack of acute myocardial infarction and present no unfavorable criteria for recovery at the time of the first examination."*

Everyone is agreed that the use of anticoagulant therapy is based on a reduction of prothrombin time by Dicumarol and related drugs. Serial determination of the prothrombin time is the only adequate method of control of such therapy and there is no substitute known at present for such control. For this reason, anticoagulant therapy with Dicumarol and related compounds (Tromexan, Link's compound 63, Danilone, etc.) should not be considered unless daily prothrombin time determinations are available. The clinical picture, bleeding times, clotting times, and other clinical laboratory procedures for studying the coagulation status of patients are totally inadequate and should not be considered tools for measuring control of therapy. The purpose of rigid control is twofold, namely, (1) reaching and maintaining effective levels, and (2) avoiding the complication of hemorrhage. Wright and associates found a level of 11-23% prothrombin activity as the optimal range for therapeutic effect, and when this range was achieved, thromboembolic phenomena were at their lowest incidence. Prothrombin percentages lower than this range produced no added advantages. Their rule of thumb was a prothrombin time which was 2½ times the control if the latter was 12-14 sec. If dosage is inadequate and optimal levels are not achieved, benefits will be minimal.

Hemorrhage is the only known complication of Dicumarol therapy. In uncontrolled cases, hemorrhage may be fatal or reach serious proportions. In cases controlled by daily prothrombin times, hemorrhage is generally insignificant. The most common clinical manifestations of hemorrhage are hematuria, hemoptysis, hematemesis, and epistaxis. Bleeding from other sources may occur and in the uncontrolled cases may involve any organ system in the body. The occurrence of hemorrhagic phenomena is a signal for cessation of anticoagulant therapy and careful following of the prothrombin time and is an indication for active treatment to restore the value toward a safe level. The treatment consists of administration of water-soluble vitamin K in a dose of 60-72 mg. intravenously once or twice. In the presence of severe bleeding, 250-500 mg. vitamin K_1 oxide orally is recommended. Transfusions of whole fresh blood or plasma may be necessary in cases associated with severe hemorrhage. Transfusions should be considered not only to re-establish the prothrombin status but also may be necessary to correct the anemia resulting from blood loss.

There is considerable difference of opinion as to the method of choice in prothrombin determinations. In clinical practice, the aim is relative simplicity with good control. A critical survey of the present knowledge of this procedure leaves us with the impression that oversimplicity is nigh impossible if one is to achieve really good control. The modified dilution methods (modified Quick, or the P and P test of Owren) appear to answer the problem of adequate laboratory control. The literature covering methods and materials is so voluminous that a review of this aspect alone would be impossible here. Suffice it to say that whatever technic is employed, certain basic principles must be adhered to: (1) the technic should be followed scrupulously as directed by the author of the technic, (2) materials should be assayed carefully for potency, and (3) controls should be included in any determination. Perhaps some of the differences in clinical results are reflections of poor laboratory control rather than an index of failure of anticoagulant therapy. One objection to insistence on such high standards of laboratory control is that hospitalization becomes almost imperative in order to achieve this. The resultant economic strain on hospitalized patients is used as an argument against routine anticoagulant therapy. However, in disease as serious as coronary occlusion it is somewhat difficult to argue against this care, except perhaps in the mildest cases. Wright[339] argues against withholding anticoagulants because it is impossible to predict which patients might develop thromboembolic phenomena. He states: "Unfortunately such prophetic vision foresight has failed. It is more difficult to

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*From Russek, H. I., and Zohman, B. I.: J.A.M.A. **156**:1130, 1954.

predict whether the course of a case will be mild or severe on the day of onset, when decisions regarding therapy must be made, than in retrospect, after the patient has been discharged from the hospital. If a physician waits until the first thromboembolic episode occurs, he may have a permanently disabled patient, as in the case of a hemiplegia due to an embolus to the brain, or a dead one."*

Dicumarol dosage is determined by routinely estimating the prothrombin activity prior to the initial dose.

If patients on anticoagulants require surgery for any reason whatsoever, the prothrombin time should be returned to normal before surgery to avoid hemorrhage.

In addition to the use of anticoagulant therapy in coronary thrombosis, it is recommended in the following conditions.

1. The majority of cases of thrombophlebitis regardless of origin, including recurrent idiopathic thrombophlebitis, postinfectious or posttraumatic. Wright[339] makes the interesting observation that thrombophlebitis that progresses relentlessly despite adequate therapeutic levels of the coumarin compounds should arouse the suspicion of malignancy. He cites 20 examples of persistence of migratory phlebitis refractory to adequate therapy. Carcinoma of the pancreas and liver are most commonly associated with distant thrombophlebitis, although other organs may also have such association.
2. The prevention of postoperative thromboembolic disease. Although statistics generally show a lower incidence of postoperative thrombophlebitis or embolus[340] in patients given prophylactic dicumarolization, there is some question as to the wisdom of such procedure **routinely,** and perhaps it should be reserved for selected cases in which there is a notoriously high incidence of postoperative thrombophlebitis, such as pelvic surgery.
3. Patients with acute embolic or thrombotic occlusion of the peripheral arteries from any of several causes.
4. Patients with recurrent embolization and atrial fibrillation such as occurs in rheumatic heart disease.
5. Postpartum patients with previous histories of recurrent thrombophlebitis.

Dicumarol therapy should be used with caution in the following conditions.

1. Severe malnutrition and cachexia.
2. Unusually prolonged menses or menorrhagia.
3. Patients with reduced prothrombin activity due to salicylates or paraaminosalicylic acid in large doses. If Dicumarol therapy appears of greater value in treating a particular patient, it is recommended withholding large dosages of these drugs which can of themselves reduce the prothrombin activity.
4. Patients under prolonged antibiotic or sulfonamide therapy in which changes in the intestinal flora affect vitamin K synthesis.
5. Severe hypertension.
6. In any patient with an initial lowered prothrombin activity unless the cause for such lowering can be well delineated and in itself does not constitute a contraindication to anticoagulant therapy.
7. In patients recently subjected to operations on the central nervous system.
8. In patients with fever. Animal experiments have shown an accentuated response in the presence of fever.

The stabilization of a patient receiving Dicumarol is not always easily accomplished because of the factors just mentioned, to which must be added the variations of absorption of an orally administered drug and the basic hypocoagulability or hypercoagulability of the plasma.

For recent reviews of the present-day concept of long-term anticoagulant therapy the reader is referred to the references at the end of this section.[341, 342]

The contraindications to anticoagulant therapy are the following.

1. **Inadequate facilities for prothrombin determinations.**
2. Patients with hemorrhagic diatheses, particularly in which prothrombin is affected directly or indirectly as a result of the disease. Hemophilia-like syndromes and the thrombocytopenic purpuras are obvious examples of such diseases. In the thrombopenic states the lowered platelet count may be primary or secondary. It is safe to say that any patient showing a defect of the coagulation mechanism should be eliminated from anticoagulant therapy.
3. Pregnancy in the second or third trimester.
4. Patients scheduled for immediate surgery.
5. Patients with moderately or fully advanced renal or liver disease.
6. Patients with jaundice due to biliary obstruction or hepatocellular disease.

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*From Wright, I. S.: Circulation 5:161, 1952.

7. Patients under continuous tube drainage of the stomach or small intestine.

8. Patients with hemorrhage of the central nervous system.

9. Patients with ulcerating lesions (gastrointestinal tract) and granulomatous lesions.

Since the introduction of anticoagulant therapy with Dicumarol, other drugs have been found and studied. Tromexan or 3,3′-carboxymethylene bis-(4-hydroxycoumarin) ethyl ester is a recent derivative and, as its chemical structure indicates, is related to Dicumarol. Compound 63 of Link[343] is a 4-hydroxycoumarin which has also been used in man and animals. Another group of anticoagulants which have come into more recent use are indanedione derivatives, such as Danilone* (2-phenyl-1,3-indanedione) and Indon† (2-phenyl-1,3-indanedione). These 2 related compounds have a more rapid effect on the prothrombin activity both in lowering the level and in returning it to normal after cessation of therapy. The hypoprothrombinemia induced by them is more refractory to vitamin K therapy than the coumarin derivatives. Excellent comparative studies of these newer anticoagulants are to be found in the *Fourth and Fifth Conferences on Blood Clotting of the Josiah Macy, Jr., Foundation.*[344, 345]

Heparin

Heparin is a mucopolysaccharide that interacts with an albumin cofactor (albumin factor X of Quick) present in the plasma to form an anticoagulant which affects all 3 phases of clotting, as previously stated. It is mainly an antithrombin, but it also has antithromboplastic and antiprothrombin effects.

Heparin is used clinically (1) to initiate anticoagulant therapy to bridge the latent period before coumarin drugs become effective, (2) in combined therapy with Dicumarol, (3) in arterial thrombosis or arteriosclerosis, and (4) in patients with hypertension requiring anticoagulant therapy. The usual dose is effective for 3-8 hr. (except Depo-Heparin‡), but the drug is cumulative. Whole blood clotting time determinations are adequate to follow heparin therapy whether regular or Depo-Heparin‡ is used.

Heparin and postoperative thrombosis and embolism: Crafoord and Jorpes of Stockholm, Sweden, utilized heparin in attempting to prevent postoperative vascular thrombosis. In 325 patients treated with heparin no great complication occurred, whereas in the control series of 1111 similar cases, complications occurred in 9%. In Sweden 30,000 doses of heparin of 50-100 mg. each have been given intravenously without any fatalities or any noteworthy drawbacks.

Murray and Best found no thrombosis or embolism in 400 patients. Moreover patients with phlebitis were improved by treatment with heparin, and favorable effects of heparin in a small number of patients with pulmonary embolism were reported.

Two developments make it mandatory for the laboratory technician to understand the action and neutralization of heparin. (1) Heparin is the anticoagulant used in open heart surgery; its concentration must be evaluated, and, if necessary, its activity must be counteracted. (2) Quite recently heparin has been used because of its lipemia-clearing action. Lipemia is associated not only with an increased clotting tendency but also with arteriosclerosis so that heparin is suggested as a treatment of arteriosclerosis and its consequences in patients with lipemia. The standard dosage should not change the whole blood clotting time.

For heparin assay method, see discussion of methods for circulating anticoagulants.

The **antidote to heparin** is protamine sulfate or Polybrene* (hexadimethrine bromide).

Plasma fibrinogen assay

The plasma fibrinogen assay methods are described on p. 1227.

THROMBOPLASTIN TESTS

Thromboplastin generation test—Biggs, MacFarlane, and Miale

Principle: This test is essentially a 1-stage prothrombin test, but the thromboplastin added is not tissue (brain) extract but intrinsic plasma thromboplastin formed by the interaction of the patient's blood components: platelets, $Al(OH)_3$-adsorbed plasma (factors V and VIII), serum (factors VII and IX), and calcium. The thromboplastic activity is tested on a substrate consisting of platelet-poor plasma which contributes prothrombin and fibrinogen. Under these circumstances, the above mixture will produce a clotting time of 10 sec. or less after 5 min. incubation at 37° C. If the thromboplastin generation is defective, the clotting time may exceed 12 sec., or although it may be shorter, it will be delayed beyond 6 min. incubation. In the case of defective thromboplastin generation, the cause can be traced to one of the 3 main components of the system—serum, adsorbed plasma, or platelets—by substituting each one at a time into a normal system. Thus the activity of the patient's fraction can be compared with the activity of the normal control fraction. For instance, by substituting a suspension of unknown platelets for normal platelets, it can be determined whether the platelet function of the unknown blood is normal or deficient.

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*Schieffelin & Co., New York, N. Y.

†Parke, Davis & Co., Detroit, Mich.

‡Upjohn Co., Kalamazoo, Mich.

*Abbott Laboratories, North Chicago, Ill.

Normal blood fractions are available commercially.*

Procedure†:

1. Collect blood using the 2-syringe silicone technic.
2. Place 3 ml. blood from the first syringe in a Kahn tube without anticoagulant but containing 3 glass beads.
3. From the second syringe, which is silicone-coated, add 4.5 ml. blood to a silicone-coated tube containing 0.5 ml. 3.8% sodium citrate and add 0.5 ml. blood to a second silicone-coated tube containing 0.5 ml. 0.1M sodium oxalate.
4. Cover each tube with Parafilm and invert repeatedly, thus ensuring clotting in the first tube and further mixing in the other 2 tubes.

Preparation of platelets and substrate plasma:

1. Centrifuge citrated blood at 1500 r.p.m. for 10 min. and then remove platelet-rich plasma.
2. Centrifuge platelet-rich plasma at 10,000 r.p.m. in an angle centrifuge for 5 min. and remove platelet-poor plasma, which furnishes the substrate plasma.
3. Suspend the remaining platelet button by stirring.

Alumina plasma:

1. Pipet 0.05 ml. $Al(OH)_3$ gel and 1 ml. platelet-poor plasma into a 13 × 100 mm. tube.
2. Mix continuously in a water bath for 3 min.
3. Centrifuge for 3 min. at 3000 r.p.m.

Patient's alumina plasma:

1. Transfer supernatant plasma to a clean test tube and label it **Alumina plasma.**
2. Determine 1-stage prothrombin time, which should be between 1 and 4 min.
3. If satisfactory, prepare a 1:5 dilution alumina plasma with saline, using 0.5 ml. alumina plasma plus 2 ml. saline.

Patient's serum:

1. 2 hr. after the blood in the tube with the glass beads is placed in a water bath, rim the clot and centrifuge for 10 min. at about 2000 r.p.m.
2. Aspirate the serum into a 13 × 100 mm. tube and label it **Patient's serum.**
3. Prepare 1:10 dilution of serum in saline solution by using 0.5 ml. serum and adding 4.5 ml. saline.

Control platelets, plasma, and serum may be obtained commercially.

Performance of test:

1. For each incubation mixture (combination of serum, plasma, and platelets) set up 6 Kahn tubes each containing 0.1 ml. substrate plasma, label 1-6, and place in a water bath at 37° C.
2. Also place in the water bath 2 tubes each containing about 4 ml. M/40 $CaCl_2$.
3. Prepare the first incubation mixture by using 0.3 ml. each of the 3 reagents (platelets, alumina plasma 1:5, and serum 1:10). The incubation mixture thus contains 0.9 ml. Place it in a water bath.
4. Add 0.3 ml. M/40 $CaCl_2$ to the incubation mixture and start an electric master interval timer. This is 0 time. Sample the mixture every min. for 6 min.
5. 1 min. after the $CaCl_2$ is added to the incubation mixture, add 0.1 ml. of the incubation mixture and 0.1 ml. $CaCl_2$ solution simultaneously with automatic pipets to the first tube of plasma substrate and start a second stopwatch immediately.
6. At 1 min. intervals, as determined from the master timer, transfer 0.1 ml. incubation mixture and 0.1 ml. $CaCl_2$ to successive tubes of substrate, starting a separate stopwatch for each tube when the incubation mixture and $CaCl_2$ are added.
7. Record the clotting time for each tube. The end point is the first appearance of a clot in each substrate tube, which is similar to the one seen in the prothrombin time determination.
8. Prepare different incubation mixtures and perform the 6 tests for each mixture.
9. Record on the worksheet the composition of the incubation mixtures, as well as the clotting times of the various tubes (Fig. 401).

Partial thromboplastin time (PTT)—Langdell et al.[349]

Materials:

1. 4.5 ml. blood obtained by clean venipuncture.
2. M/10 Sodium oxalate.
3. Partial thromboplastin.

   This may be crude cephalin or Asolectin.* The cephalin is prepared as a 0.3% suspension, the Asolectin as a 1% suspension. Adjust by dilution in saline to give a clotting time range of 40-80 sec. with normal plasma.

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*TGTR; Warner-Chilcott Laboratories, Morris Plains, N. J.

†Method described is based partly on the description of the method for TGT in Mandel, E. E.: Workshop on techniques in blood coagulation, Chicago, 1960, American Society of Clinical Pathology, and partly on Miale, J. B.: Coagulation workshop book, Kansas City, 1959, American Society of Clinical Pathology.

*Commercial preparation made from crude soya lecithin; Associated Concentrates, Division American Lecithin Co., Inc., Long Island, N. Y.

Incubation mixtures			Clotting times (sec.)					
$Al(OH)_3$ plasma + 1:5	Serum + 1:10	Platelet or plasma reagent						
Normal	Normal	Normal						
Normal	Patient	Normal						
Patient	Normal	Normal						
Normal	Normal	Patient						
Patient	Patient	Patient						

Fig. 401. Worksheet for thromboplastin generation test.

4. 0.02M Calcium chloride.

Procedure: The procedure is essentially that of the 1-stage prothrombin activity test.

1. Add 4.5 ml. venous blood to 0.5 ml. M/10 sodium oxalate.
2. Separate the plasma by centrifugation.
3. Keep all reagents at 37° C.
4. Mix in the following amounts and order: 0.1 ml. plasma, 0.1 ml. partial thromboplastin, and 0.1 ml. 0.02M calcium chloride.
5. Record the time in sec. for clotting to occur.

Normal: Normal values depend on concentration of thromboplastin used and are usually 40-80 sec.

Comments: The principle is that partial thromboplastins give a longer clotting time than complete thromboplastins, such as are used in routine prothrombin determinations. Langdell et al. found that complete thromboplastins clot normal and hemophiliac bloods equally fast. However, partial thromboplastins clot hemophiliac bloods less rapidly than normal bloods.

This test has been offered as a useful screening procedure in hemophiliac states.[280] It may be substituted for the prothrombin consumption tests described on p. 1207. Partial thromboplastin is available commercially[*]; therefore this test can be used more readily as a screening procedure for latent bleeders.[350]

Hicks-Pitney modification of TGT

This is a rapid simplified modification of the TGT which allows routine screening. Two commercial reagents, Platelin[†] and Diagnostic Plasma,[†] are required.

Reagents:

1. Platelin.
2. Diagnostic Plasma.
3. 0.025M Calcium chloride.
4. 0.1M Sodium oxalate or citrate.
5. Sodium chloride, 0.85%.

Preparation of reagents:

1. Reconstitute the Platelin by adding 2.5 ml. distilled or deionized water to the vial. Shake to ensure dispersion of the fine suspension.
2. Reconstitute the Diagnostic Plasma by adding 0.5 ml. distilled water. Swirl gently to dissolve. For multiple determinations, the large bacteriologic sized vial may be used: reconstitute with 2.5 ml. distilled water.
3. Dilute the patient's plasma to 10% by adding 0.1 ml. patient's plasma to 0.9 ml. 0.85% saline solution. Testing should be started within 5 min. of the dilution.

Procedure: Place the following in a water bath at 37° C.: test tube containing patient's diluted plasma, test tube containing Platelin, test tube containing 2 ml. 0.025M calcium chloride, empty test tube, 4 prothrombin time tubes, each containing 0.1 ml. 0.025M calcium chloride, and test tube containing Diagnostic Plasma.

The entire test is done in a 37° C. water bath. Two timers are required: one to measure the overall incubation period and the other for the individual prothrombin time determinations.

1. Into the empty test tube pipet in rapid succession 0.2 ml. each Platelin, patient's dilute plasma, and 0.025M $CaCl_2$. Start the incubation timer simultaneously with the addition of the $CaCl_2$. This is the generation mixture.
2. At 3 min. 45 sec., remove 0.1 ml. generation mixture and blow into one of the tubes containing 0.1 ml. 0.025M calcium chloride. **Do not** stop the incubation timer.
3. At 4 min., blow 0.1 ml. Diagnostic Plasma into the tube containing 0.1 ml. each of generation mixture and calcium chloride. Simultaneously, start the second timer and time the clot formation exactly as in the prothrombin time test.
4. At 5 min. 45 sec. repeat step 2.
5. At 6 min. repeat step 3.
6. At 7 min. 45 sec. repeat step 2.
7. At 8 min. repeat step 3.
8. At 9 min. 45 sec. repeat step 2.
9. At 10 min. repeat step 3.

When enough plasma thromboplastin has been generated to clot the Diagnostic Plasma in 16 sec. or less, stop the incubation timer and terminate the test.

Note: If a clot forms in the generation mixture tube at any time during the test, remove it to prevent interference with pipeting. **Do not** stop the incubation timer (a clot in the generation mixture has no bearing on the test).

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[*] Ortho Pharmaceutical Corp., Raritan, N. J.

[†] Warner-Chilcott Laboratories, Morris Plains, N. J.

Results: If stage 1 is normal, incubating the generation mixture for 8-10 min. will generate enough plasma thromboplastin to give a 16 sec. prothrombin time with Diagnostic Plasma. If the Diagnostic Plasma does not clot in 16 sec. or less after 10 min. incubation of the generation mixture, one of the factors necessary to stage 1 of coagulation (plasma thromboplastin formation) is probably deficient in the patient's plasma. To pinpoint the specific defect, a TGT should be done and the results are interpreted according to the more definitive TGT.

TESTS FOR CIRCULATING ANTICOAGULANTS

Heparin assay—Quick[263]

Principle: In plasma, heparin acts as an antithrombin. If heparin is added to a standard solution of thrombin in oxalated plasma, the clotting time becomes proportional to the concentration of heparin.

Reagents:

1. Normal control blood.
   (a) Obtain blood by 2-syringe silicone technic and prepare oxalated plasma.
2. Thrombin, full strength.
   (a) Add 0.1 ml. thrombin to 0.2 ml. fresh oxalated plasma so that a clotting time of about 3 sec. is achieved.
3. Heparin.
   (a) Dissolve 100 mg. heparin in 10 ml. NaCl.
   (b) Add 0.25 ml. this solution to 50 ml. distilled water so that 1 ml. this solution contains 50γ heparin or that 0.01 ml. solution equals 0.5γ heparin.
4. Protamine sulfate.
   (a) Prepare 0.5% solution in 0.85% NaCl or 1% toluidine blue solution in distilled water.

Procedure:

1. Add 0.01 ml. heparin solution to 0.5 ml. fresh oxalated plasma.
2. After 5 min. incubation at 25° C., transfer 0.2 ml. this plasma to another test tube and incubate at 37° C. for 15 sec.
3. Blow 0.1 ml. thrombin solution into this plasma and determine the clotting time.
4. Repeat the test with 0.02, 0.03, 0.04, and 0.05 ml. heparin solution.

Average results are given in Table 242. These figures act as a base line for the following determinations.

Table 242

Heparin solution (ml.)*	0	0.01	0.02	0.03	0.04	0.05
Heparin concentration (γ)		1	2	3	4	5
Clotting time (sec.)	7	9	12	16	22	30

*Added to 0.5 ml. plasma.

Determination of heparin (or heparin-like substances) in blood[263]

To 0.2 ml. of patient's oxalated plasma, add 0.1 ml. thrombin solution and 0.01 ml. of physiologic saline solution. The control clotting time for this system was 9 sec. (see Table 242). If, for example, the patient's clotting time is 30 sec., then it must be determined whether the inhibitory substance is heparin by neutralizing it with protamine.

To 0.2 ml. patient's oxalated plasma, add 0.1 ml. thrombin solution and 0.01 ml. protamine sulfate (or toluidine blue solution). If in this system the clotting time now returns to 9 sec., the patient's blood contains 5 μg heparin.

Fibrinolysis

Simple screening test for increased fibrinolysis—Stefanini-Dameshek

Equipment: The equipment is the same as that used for thrombin time, plus purified bovine fibrinogen, 600 mg./100 ml. saline (optional).

Procedure:

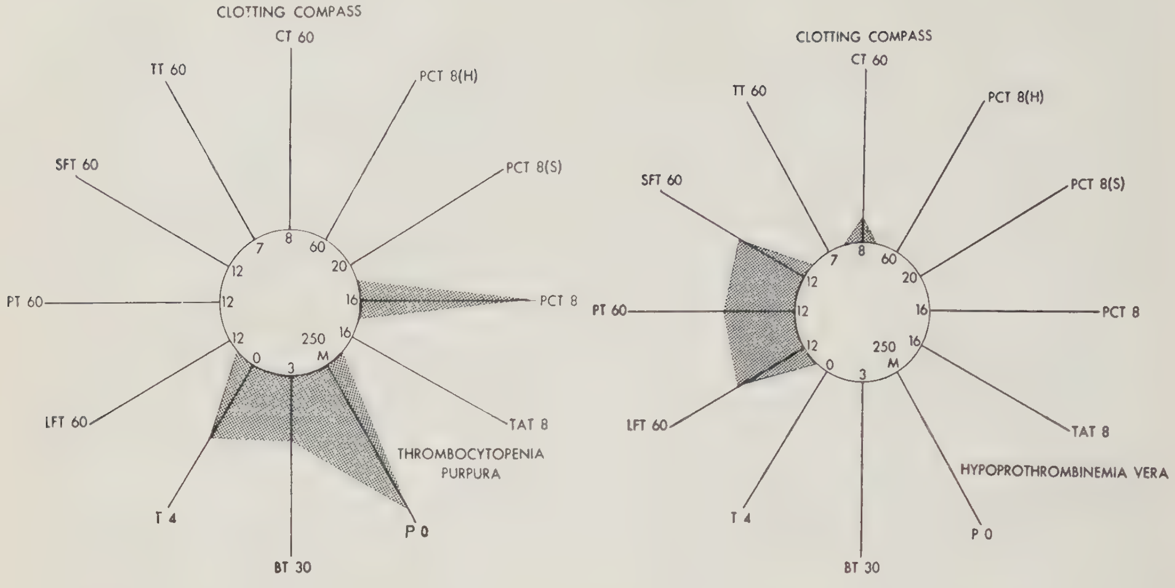
1. Prepare platelet-poor plasma from citrated venous blood collected from both the patient and a control subject by adding 4.5 ml. venous blood to 0.5 ml. 3.8% sodium citrate. Centrifuge slowly for 10 min., obtain platelet-rich supernatant fluid, and centrifuge this at high speed for 30 min.
2. Maintain ice-water temperature from the moment of venipuncture and allow no delay between steps in procedure.
3. From each of the 2 plasmas transfer 0.5 ml. to a cold test tube and mix. In addition, place 1 ml. each plasma in separate tubes.
4. Transfer all 3 tubes from the ice bath to a 37° C. bath, add to each 0.1 ml. saline solution containing 10 units of thrombin, and mix immediately.
5. Observe each clot at frequent intervals (at least every 30 min. for the first 6 hr.) for detection of complete lysis.

Normally no noticeable lysis occurs in 24 hr. Lysis time can be shortened and the test can be made more convenient by substituting for normal plasma purified bovine fibrinogen, which contains little antifibrinolysin.

The assay of fibrinolytic activity will be an important test in the future if fibrinolytic agents such as human fibrinolysin or streptokinase prove successful in the treatment of recent thromboses. Experimental methods are suggested in the references.[351-353]

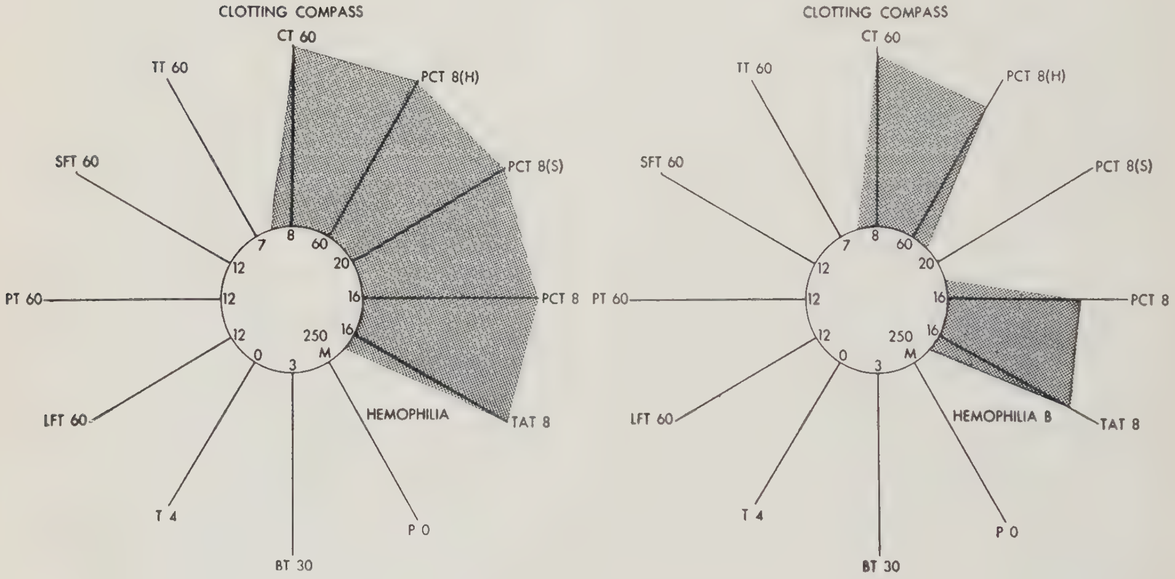
CLOTTING COMPASS

On the basis of the coagulogram of Gerendas, Quick[354] designed the clotting compass (Figs. 402 and 403) to provide a diagrammatic presentation of the clotting defects in various hemorrhagic diseases. This approach allows easy visualization and ready comparison of the clotting pattern and consists of the various tests described in the preceding pages.



A B

Fig. 402. **A,** Clotting compass in thrombocytopenic purpura. **B,** Clotting compass in hypoprothrombinemia. (From Quick, A. J.: Thromb. diath. haemorrhag. **3**:302, 1959.)



A B

Fig. 403. **A,** Clotting compass in hemophilia. **B,** Clotting compass in hemophilia B. (From Quick, A. J.: Thromb. diath. haemorrhag. **3**:302, 1959.)

The patterns of some of the hemorrhagic disorders are reproduced. The lines representing the tests radiate from an inner circle corresponding to the normal values of the methods. The distal points in the periphery represent the maximal abnormal values. The following abbreviations are used.

CT = clotting time
PCT(H) = prothrombin consumption time with hemolysate
PCT(S) = prothrombin consumption time with serum
PCT = prothrombin consumption time
TAT = thromboplastinogen activity time
P = platelet count
BT = bleeding time
T = tourniquet test
LFT = labile factor time
PT = prothrombin time
SFT = stable factor time
TT = thrombin time

HEMOSTASIS AND ABNORMALITIES OF BLOOD COAGULATION

In the normal individual, blood courses through the vascular tree in a properly fluid state, carrying a multiplicity of substances and structures. Life itself is dependent upon the complex regulatory mechanisms that maintain an exquisite balance of all the factors which combine to make for this state of normality. A basic requirement for sustaining such a status quo is an intact vascular system which, while maintaining its integrity, still permits selective exchanges of vital materials between the circulatory system and the tissues. Although a great deal of knowledge has been accumulated concerning capillaries, there is perhaps more information concerning their reaction to injury and in disease than answers as to why they remain intact in the normal individual. Alterations in the character of the vascular bed may occur as the result of a multiplicity of causes, involving direct and indirect assault on the vessels themselves or on the contents of the vessels. The end result of such attacks may be the escape of blood from the vessels or the closure of vessels. It is quite apparent from these remarks that the study of any patient in whom the defense mechanisms for maintaining the normal liquid state of blood in an intact vascular system have been threatened involves consideration of many factors. These might be conveniently considered under the following general headings: (1) the state of the vascular tree, (2) the state of the coagulation mechanism, and (3) the status of tissues and organs related to the elaboration of elements essential to maintaining normal coagulability of blood, notably the liver, spleen, and bone marrow.

Alterations in one or in combinations of these basic states may result in either hemorrhagic diathesis or thrombosis. The clinical results of such derangements cover the entire spectrum from mild symptoms to fatal outcome. In the study of patients with any such symptoms, consideration of the causes involves complete history, physical examination, and laboratory examination. As in all clinical medicine, we cannot emphasize too strongly the need for thorough history taking and physical examinations **before** rushing to the laboratory for answers to such patients' problems. This orderly procedure will often circumscribe the disease process and eliminate the necessity of costly and time-consuming laboratory tests.

The **history** should completely consider the family history as well as the personal history of the patient. In hemorrhagic diseases, hemophilia is undoubtedly the outstanding example. As knowledge of the factors involved in hemostasis has grown, many other examples of hereditary disease have been uncovered. Following is a list of diseases in which heredity plays a role.*

HEMORRHAGIC DISEASES

Hereditary hemorrhagic diseases

Capillary purpura due to congenital abnormalities of vascular wall
  Hereditary capillary fragility (pseudohemophilia, purpura of capillary weakness, von Willebrand's disease)
  Hereditary capillary telangiectasia (Rendu-Weber-Osler disease, Goldstein's heredofamilial angiomatosis, hereditary angiomata, hereditary hemorrhagic telangiectasia)

Thrombocytopenic purpura due to
  Hereditary thrombocytopenia
  Thrombocytopathic purpura
  (Glanzmann's thrombasthenia)
  Hegglin's syndrome

Plasma defects affecting coagulation due to congenital deficiencies
  Congenital hypoprothrombinemia
  Congenital fibrinogenopenia
  Congenital deficiency of antihemophilic globulin (AHG)

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*This list is based on the recommended nomenclature published by a committee sponsored by the American Medical Association and the American Society of Clinical Pathologists (Am. J. Clin. Path. 20:575, 1950). The designations appearing in parentheses are synonymous terms found in the literature but which this committee recommends avoiding. Minor additions to the original list have been made by the author.

- Congenital deficiency of plasma thromboplastin component (PTC)
- Congenital deficiency of plasma thromboplastin antecedent (PTA)
- Congenital deficiency of accelerator factor (factor V deficiency, labile factor deficiency, plasma Ac-globulin deficiency, parahemophilia)

In the personal history of the patient suffering from one of the hemorrhagic diatheses, many possibilities of acquired disease other than obvious trauma are to be considered. For the purpose of orientation as to the multiplicity of these factors, the following list has been compiled.

- Capillary purpura due to increased vascular permeability and fragility
  - Mechanical cause
  - Infection (toxic purpura, Waterhouse-Friderichsen syndrome)
  - Emboli
  - Drugs
  - Allergy (anaphylactoid) (Schönlein's purpura, purpura abdominalis, Henoch's purpura, anaphylactoid purpura, purpura rheumatica, peliosis rheumatica, hemorrhagic capillary toxicosis)
  - Metabolic disturbances
    - Anoxia
    - Vitamin deficiency (scorbutic purpura, rutin deficiency)
    - Uremia
    - Senility
    - Endocrine (Ehlers-Danlos syndrome?)
  - Neoplasms
    - Leukemia
    - Hemorrhagic sarcoma (Kaposi's sarcoma)

- Thrombocytopenic purpura (Werlhof's disease, purpura hemorrhagica, primary or essential thrombocytopenia) due to
  - Idiopathic thrombocytopenic purpura (ITP)
  - Thrombotic thrombocytopenia (Moschcowitz's syndrome)
  - Thrombocytic hypoplasia from endocrine disturbance or irradiation
  - Drugs
  - Allergy
  - Myelophthisis from unknown cause, bone marrow replacement by tumor, leukemia, fibrous tissue, etc., or bone marrow aplasia (hypoplastic and aplastic anemias)
  - Infection

- Splenic disease or hypersplenism due to
  - Congestion
  - Lipid histiocytosis
  - Sarcoidosis
  - Tuberculosis
  - Neoplasm, primary or secondary
  - Leukemia
  - Liver disease
  - Acquired antibodies
- Thrombocytopenia associated with congenital hemangioma
- Qualitative platelet deficiencies (thrombocytoasthenia)
- Nutritional deficiencies, e.g., pernicious anemia

- Plasma defects affecting coagulation, acquired
  - Hypoprothrombinemia
    - Vitamin K deficiency
      - Of newborn (melena neonatorum, hemophilia neonatorum, morbus hemorrhagica neonatorum)
      - Deficient absorption from gastrointestinal tract
      - Obstructive jaundice
      - Steatorrhea
      - Drugs
    - Liver disease (pseudohemophilia hepatica)
    - Drugs, particularly Dicumarol (sweet clover disease)
  - Fibrinogenopenia due to liver disease
  - Endogenous anticoagulant
    - Drugs, especially heparin
    - Leukemia
    - Radiation
    - Venoms of snakes

- Circulating anticoagulants
  - Antithromboplastin
  - Antithrombin
  - Increased fibrinolysis

- Dysproteinemias
  - Multiple myeloma
  - Macroglobulinemia
  - Amyloidosis
  - Hyperglobulinemia

Although this list appears formidable, an adequate history oriented along these lines may be very fruitful. Wearing of tight garters or other restraining garments may be the source of mechanical purpura. At times the investigation need go no farther than observation of such patients without this apparel. The complete disappearance of symptoms on this regimen is generally sufficient proof of the causal factor.

Hemorrhagic diseases due to vascular defects

Vascular defects are 2-fold: increased permeability, such as occurs in vitamin C deficiency (ground substance defect) or increased fragility, which may be congenital or related to platelet deficiency or plasma factor deficiencies. Whatever the etiology there must be a vascular defect before bleeding from a blood vessel can occur.

For the purpose of description and classification it is best to separate the purely vascular purpuras from those caused by platelet or plasma factor deficiencies, though in some cases there may be some overlap.

Congenital vascular abnormalities

Congenital vascular abnormalities include hereditary hemorrhagic telangiectasia and pulmonary hemosiderosis. The latter is described in the discussion of iron-deficiency anemias.

The first disease shows dilatation and thinning of patchy collections of capillaries, frequently leading to bleeding from the nose and lung though bleeding may occur from the gastrointestinal and genitourinary tract.

Increased vascular permeability

Scurvy: Purpura is produced by increased vascular permeability due to vitamin C deficiency resulting in defective endothelial cement substance. Clinically important is the gingival bleeding which may point toward this condition. Vitamin P (hesperidin) deficiency may also lead to capillary oozing.

Increased vascular fragility

Some of the most frequently seen hemorrhagic tendencies are included in this classification. Ecchymoses and purpuric spots appear in the skin and frequently spare the mucous membranes. In simple hereditary purpura, in senility, in some endocrine disturbances such as Cushing's disease, cortisone administration, and menopause, in the course of infections, in toxemias such as uremia and diabetes, and after prolonged standing (orthostatic), the vessel walls are damaged and allow blood to escape. Similar damage may result from drug administration (see discussion of thrombocytopenias). Schönlein-Henoch purpura is an allergic or infectious form of purpura which has received special attention.

Schönlein-Henoch purpura

This disease is also referred to as allergic purpura or nonthrombocytopenic purpura. It is characterized by an urticaria-like eruption associated with petechiae and gastrointestinal symptoms (Henoch's purpura). It may also be accompanied by painful swelling of the joints (Schönlein's purpura rheumatica) and involvement of the kidneys. As the individual case may combine features of both, the disease is usually called Schönlein-Henoch purpura. Although the allergic nature of the disease is generally accepted, it appears to be extremely difficult in an individual case to discover the allergen. The allergen may be a bacterial toxin (streptococcal ?) or foods such as eggs, milk, and chocolate. Clinically important are the renal complications which produce a picture similar to that seen in glomerulonephritis or in some of the collagen diseases.

Laboratory findings in vascular defects

Bleeding time: Prolonged.
Coagulation time: Normal.
Clot retraction: Normal.
Tourniquet test: Positive.
Prothrombin time: Normal.
Prothrombin consumption time: Normal.
See also discussion of hematologic methods.

DEFECTS IN THROMBOCYTOPOIESIS

Both the increase (**thrombocythemia**) and the decrease (**thrombocytopenia**) in the number of platelets may be accompanied by hemorrhagic phenomena. Evidence of bleeding may also be seen when the platelets, though normal in number, are qualitatively inadequate (**thrombocytoasthenia**). In Chapter 62 conclusive proof was offered that the megakaryocytes of bone marrow produce platelets. Platelet changes may be associated with normal, decreased, increased, and qualitatively altered megakaryocytes.

Thrombocythemias

An increase in platelets is always associated with an increase in megakaryocytes (**megakaryocytosis**). As previously discussed, variations in the number of megakaryocytes are better appreciated in bone marrow sections than in bone marrow smears. Two types of thrombocythemias can be distinguished: (1) primary thrombocythemia, which may be seen in such myeloproliferative disorders as chronic myelogenous leukemia, polycythemia, myelofibrosis, and essential thrombocythemia (platelet or megakaryocytic leukemia) and (2) secondary thrombocythemia, which may be seen in multiple thrombosis, agranulocytosis, after hemorrhage, and after splenectomy.

Primary thrombocythemia

There is a marked variation in size of platelets from microplatelets to giant platelets and an increase in the number of thrombocytes up to 1 million or more, associated with a moderate rise in the total white and red blood cell counts. On clinical examination, splenomegaly is noted. There is a tendency to both thrombus formation on the one hand and bleeding on the other. The hemorrhagic phenomena are due to qualitative platelet changes. These changes may include a platelet factor 3 deficiency, or some difficulty in platelet disintegration (pachyderm platelets), or both, so that the thromboplastin generation test is abnormal when the patient's platelets are utilized. Phase microscopy examination of the platelets reveals absence of pseudopod formation and spreading, suggesting an intercellular enzymatic defect. The bone marrow shows a marked increase in normal to ab-

Table 243. *Hemorrhagic diseases**

Term to be used	*Terms to be avoided*
Capillary purpura due to	Symptomatic purpura, nonthrombopenic purpura, purpura simplex, purpura fulminans, erythema simplex
Unknown cause	
Mechanical cause	
Infection	Toxic purpura, Waterhouse-Friderichsen syndrome
Emboli	
Drugs	
Allergy	Schönlein's purpura, purpura abdominalis, Henoch's purpura, and anaphylactoid purpura, purpura rheumatica, peliosis rheumatica, hemorrhagic capillary toxicosis
Metabolic disturbance	
Anoxia	
Vitamin deficiency	Scorbutic purpura
Uremia	
Senility	
Endocrine	Purpura cachectica
Hereditary capillary fragility	Pseudohemophilia, purpura of capillary weakness, von Willebrand's disease
Hereditary capillary telangiectasia	Rendu-Weber-Osler disease, Goldstein's heredofamilial angiomatosis, hereditary angiomata, hereditary hemorrhagic telangiectasia
Neoplasms	
Leukemia	
Hemorrhagic sarcoma	Kaposi's sarcoma
Thrombocytopenic purpura due to	Morbus maculosis hemorrhagicus, Werlhof's disease, purpura hemorrhagica, primary or essential thrombocytopenia
Unknown cause	Hemogenia, hemogenic syndrome, idiopathic thrombocytopenia
Idiopathic thrombocytopenic purpura	
Hereditary thrombocytopenia	
Congenital thrombocytopenia	
Thrombotic thrombocytopenia	Moschcowitz's syndrome
Thrombocytic hypoplasia, from	
Endocrine disturbance	
Irradiation	
Drugs	
Allergy	
Myelophthisis from	
Unknown cause	
Infection	
Splenic disease	
Congestive	
Lipid histiocytosis	
Sarcoidosis	
Leukemia	
Thrombocytopathic purpura	Glanzmann's thrombasthenia
Thrombocytosis due to	Thrombocythemia
Unknown cause	
Idiopathic thrombocythemia	
Polycythemia vera	
Neoplasm	
Leukemia	
Splenectomy	
Plasma defects affecting coagulation due to	
Unknown cause	Hereditary thrombasthenia, Glanzmann's thrombasthenia, constitutional thrombopathy, hereditary pseudohemophilia
Hypoprothrombinemia	Hemorrhagia universali
Unknown cause	
Vitamin K deficiency of newborn	Melena neonatorum, hemophilia neonatorum, morbus hemorrhagica neonatorum
Deficient absorption	
Obstructive jaundice	
Steatorrhea	
Drugs	
Liver disease	Pseudohemophilia hepatica
Familial	
Drugs	
Dicumarol	Sweet clover disease
Fibrinogenopenia due to	Hypofibrinogenemia, pseudohemophilia, afibrinogenemia
Unknown cause	
Liver disease	Pseudohemophilia hepatica
Heredity	

*From Am. J. Clin. Path. **20**:574, 1950.

Table 243. *Hemorrhagic diseases*—cont'd

Term to be used	*Terms to be avoided*
Deficiency of accelerator factors due to	Factor V deficiency, labile factor deficiency
Familial	Parahemophilia
Hemophilia	
Hereditary	
Sporadic	
Endogenous anticoagulant	
Unknown cause	
Drugs	
Leukemia	
Radiation	

normal megakaryocytes surrounded by thick coats of various-sized platelets.

There may be some difficulty in distinguishing this platelet disease from forms of myeloproliferative disorders associated with a marked increase in platelets.

Thrombasthenia

Glanzmann (1918) first described a case with a normal number of platelets but with qualitative abnormalities leading to hemorrhagic manifestations. Electron microscopic examination of these platelets again shows a defect in pseudopod formation and in spreading in some cases, whereas other cases show excessive spreading. Abnormal viscous metamorphosis has also been noted, as well as diminution in certain enzymes such as glyceraldehyde phosphate dehydrogenase and pyruvatekinase. Secondary thrombasthenia has been reported in liver disease.

Thrombocytopenia[360-365]

Clinically thrombocytopenia leads to petechial hemorrhages involving the skin and mucous membranes so that internal hemorrhages are a frequent complication.

Three general classifications are distinguished: (1) thrombocytopenia due to defects in platelet production (amegakaryocytic form of Dameshek), (2) thrombocytopenia due to defects in platelet maturation, and (3) thrombocytopenia due to excessive peripheral destruction of platelets (megakaryocytic type of Dameshek).

Defects in platelet production

Amegakaryocytic thrombocytopenia

The following defects in platelet production are included in this type of thrombocytopenia:

- Congenital absence of megakaryocytes
- Bone marrow failure in aplastic anemia
- Bone marrow replacement by carcinoma, leukemia, lymphomas, etc.
- Toxic depression of megakaryocytes in severe infections and radiation therapy and by drugs (benzol, quinidine, etc.) acting directly on megakaryocytes

Defects in platelet maturation

Defects in platelet maturation occur in the following conditions:

- Pernicious anemia, which produces abnormal megakaryocytes and platelets
- Hegglin's syndrome, which involves polymorphonuclear cells and platelets
- Hypersplenism producing splenic thrombocytopenia either due to hormonal action or to antibodies, which act on platelets or megakaryocytes
- Congenital afibrinogenemia associated with thrombocytopenia
- Aldrich's syndrome (thrombocytopenia, eczema, and recurrent infection in infants and children)
- Paroxysmal nocturnal hemoglobinemia

Excessive destruction of platelets

Immune thrombocytopenias

Immune thrombocytopenias may be classified according to clinical course or according to etiology, as follows:

- *Classification according to clinical course*
  - Acute
  - Chronic
- *Classification according to etiology*
  - Allergic reactions
    - Drugs: quinine, quinidine, Sedormid, sulfonamides, digitoxin, mercury compounds, and codeine
    - Bacterial and viral infections
    - Waterhouse-Friderichsen syndrome
    - Dysproteinemias: lupus erythematosus and other collagen diseases
    - Hypersplenism
    - Hemolytic anemias
    - Idiopathic conditions: idiopathic thrombocytopenic purpura, isoimmune mechanism following multiple blood transfusions and during pregnancy and neonatal thrombocytopenia

Recently Cohen and others have reclassified thrombocytopenia by the Cr^{51}-labeling method for measuring platelet life span in newly diagnosed patients who did not receive transfusion, as follows:

Classification according to life span of Cr^{51}-labeled platelets

- Primary production deficit with normal life span
  - Various types of Dameshek's amegakaryocytic thrombocytopenia
  - Paroxysmal nocturnal hematuria
- Production deficit predominates but some shortening of life span present
  - Defects in platelet maturation of preceding classification
  - Nonimmune idiopathic thrombocytopenia
- Peripheral destruction predominates but relative production deficit sometimes present
  - Associated with megakaryocytic hyperplasia
    - Acute childhood immune idiopathic thrombocytopenia
    - Acute and chronic adult immune idiopathic thrombocytopenia
    - Immune thrombocytopenia secondary to lupus erythematosus
    - Drug-induced thrombocytopenia (quinidine-Sedormid prototype)
    - Compensated thrombocytopenia
      - Postsplenectomy idiopathic thrombocytopenic purpura
      - Postsplenectomy Felty's syndrome
      - Corticosteroid remission in idiopathic thrombocytopenic purpura
    - Granulomatous disease of the spleen
      - Sarcoidosis
      - Tuberculosis
    - Reticuloendothelial hyperplasia of the spleen not caused by portal hypertension
      - Felty's syndrome
      - Infectious mononucleosis
      - Acquired idiopathic hemolytic anemia (Evan's syndrome)
    - Myeloid metaplasia with massive splenomegaly
    - Thrombotic thrombocytopenic purpura
  - Associated with normal or reduced bone marrow megakaryocytes
    - Lymphosarcoma
    - Chronic granulocytic leukemia with massive splenomegaly
    - Thrombotic thrombocytopenic purpura

Platelets are proteinaceous and therefore antigenic. They contain blood group substances A and B, as well as up to 8 serologically different platelet antigens. There is some evidence that the contents of the A and B blood groups is simply adsorbed on the surface of the platelets.

Antibodies involved in immune thrombocytopenia may be shared with other cellular elements (red blood cells, white blood cells, etc.), they may be specific platelet antibodies, or they may be isoantibodies, immune antibodies, or autoantibodies. All may be either warm or cold in type.

Immune mechanisms

Several types of immune mechanisms may be responsible for platelet destruction. In the thrombocytopenia following administration of drugs or infections, it is thought that a hapten combines with the platelets to form an antigenically active hapten-platelet complex. These antigenically modified platelets agglutinate and are destroyed.

It is also possible that the hapten attaches itself to a plasma protein, and it is this hapten-plasma-protein complex which is antigenically active. If the patient is re-exposed to the original drug or infecting organism, the antibody attaches itself to the platelets, which are thus altered or destroyed.

Damage to the platelets may result from antigen-antibody reactions not primarily directed against platelets but primarily involving other cellular elements. This reaction which requires complement or an additional plasma factor, at least experimentally, leads to the release of histamine and of 5-hydroxytryptamine. This type of mechanism may be responsible for the symptomatic thrombocytopenias secondary to other diseases, e.g., leukemia, collagen diseases, etc.

Last, the antibody may be a specific platelet antibody acting on normal platelets. The thus sensitized platelets are agglutinated and destroyed by the reticuloendothelial system. In the presence of complement, they may be lysed. This mechanism is probably operative in isoimmune thrombocytopenias (following blood transfusions, etc.).

The platelets, damaged by the immune mechanism, are destroyed in the spleen. There is some evidence that the antiplatelet antibodies may also have some anticapillary or antiendothelial action which would be responsible for the purpura. Some of the platelet antibodies may also act directly on the megakaryocytes in the bone marrow because they share certain antigens with the platelets and damage them.

Idiopathic thrombocytopenic purpura (ITP)

Clinically idiopathic thrombocytopenic purpura is divided into an acute form which most frequently occurs in children and into a chronic form which is more common in adults. Both forms have in common thrombocytopenia in peripheral blood and normal or increased megakaryocytes in bone marrow, most of which are not platelet-producing.

Acute idiopathic thrombocytopenic purpura: This disease occurs most frequently in children who suddenly break out with petechiae involving the skin and mucous membranes. The platelet count is extremely low, less than 10,000, but the platelet morphology is more or less normal. Eosinophilia and lymphocytosis may be present. The bone marrow contains the usual number of megakaryocytes, most of which are young, are not platelet-producing, and show evidence of degeneration.

Chronic idiopathic thrombocytopenic purpura: As the name implies, this disease may last for years, interrupted by remissions. The platelet count is moderately low, less than 50,000. The bone marrow contains an increased number of mature megakaryocytes which show diminished platelet production. Platelet agglutinins are present in about 50% of the cases.

Pathogenesis of idiopathic thrombocytopenic purpura: For many years some splenic factor acting on the bone marrow was considered to be the etiologic agent since splenectomy frequently leads to an improvement in the clinical condition and the bone marrow. In recent years the immunoallergic theory has found more and more supporters. In about 50-70% of the cases of idiopathic thrombocytopenic purpura, Harrington has proved the existence of antithrombocytic antibodies, agglutinins, and lysins.

Harrington[355] has summarized the clinical significance of the antibodies for platelets, pointing out (1) that these agglutinins play a major role in the production of the thrombocytopenic state, (2) that platelet agglutinins may appear after repeated transfusions, and (3) that platelet agglutinins may appear as a sequela of pregnancy. The initial phase of Harrington's research in this field is a dramatic story which has opened the door to a brilliant research problem. To prove that the plasmas of patients with idiopathic thrombocytopenic purpura have a plasma factor which will induce thrombocytopenia, he had injected into himself such plasma and promptly experienced the clinical and hematologic picture of thrombocytopenic purpura.

From this beginning, the experiments have been elaborated both in vivo and in vitro. In 72 patients with idiopathic thrombocytopenic purpura, he found 50 who showed positive agglutinins and 22 who were negative for agglutinins. In the acute cases 30 out of 43 were positive, and in the chronic variety 20 out of 29 were positive for agglutinins. Auto-

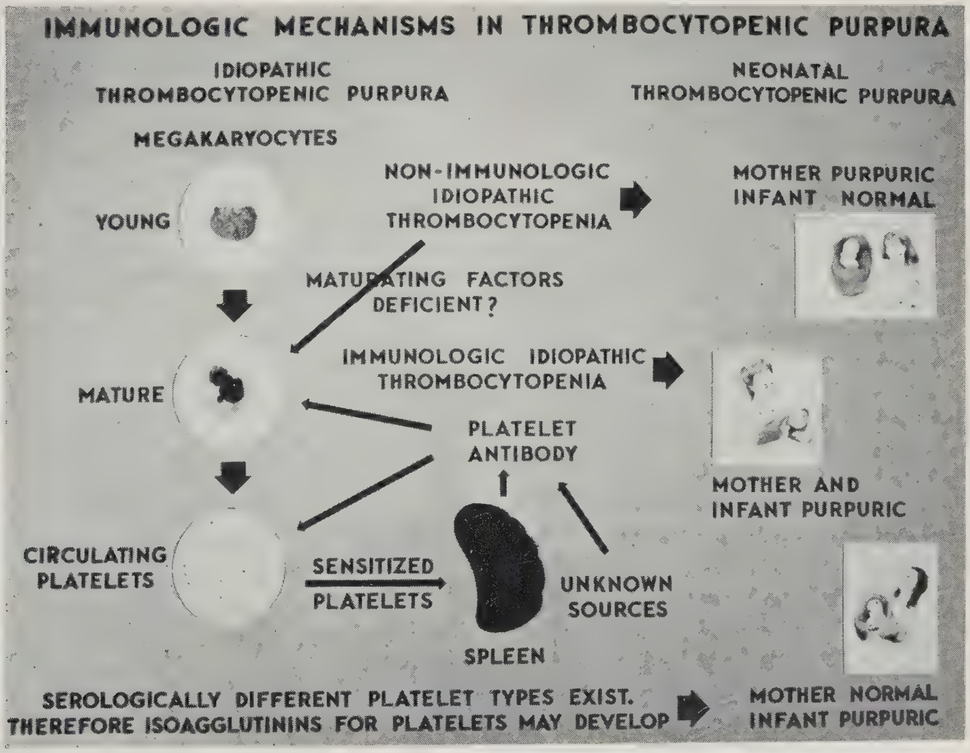
IMMUNOLOGIC MECHANISMS IN THROMBOCYTOPENIC PURPURA
IDIOPATHIC THROMBOCYTOPENIC PURPURA
NEONATAL THROMBOCYTOPENIC PURPURA
MEGAKARYOCYTES
YOUNG
NON-IMMUNOLOGIC IDIOPATHIC THROMBOCYTOPENIA
MOTHER PURPURIC INFANT NORMAL
MATURATING FACTORS DEFICIENT?
MATURE
IMMUNOLOGIC IDIOPATHIC THROMBOCYTOPENIA
PLATELET ANTIBODY
MOTHER AND INFANT PURPURIC
CIRCULATING PLATELETS
SENSITIZED PLATELETS
UNKNOWN SOURCES
SPLEEN
SEROLOGICALLY DIFFERENT PLATELET TYPES EXIST. THEREFORE ISOAGGLUTININS FOR PLATELETS MAY DEVELOP
MOTHER NORMAL INFANT PURPURIC

Fig. 404. Pictorial presentation of current concepts of the pathogenesis of idiopathic and neonatal thrombocytopenic purpura. (From Harrington, W. J.: Sang 25:712, 1954.)

agglutinins were not tested in all of these cases because in some instances only serum was available, but when both platelets and serum of the patients were available, two-thirds of the cases (27) showed autoantibodies. In addition to idiopathic thrombocytopenic purpura, autoantibodies were demonstrated in 2 cases of chronic lymphocytic leukemia, 2 cases of lupus erythematosus disseminata, a case of carcinomatosis, a case of atypical acquired hemolytic anemia, and a case of drug sensitivity. These cases represent 7 out of 13 controls with secondary thrombocytopenia in whom it was possible to test for autoantibodies. In this group of secondary thrombocytopenias, 28 out of 57 patients tested showed positive agglutinins in their sera. However, these agglutinins included both isoagglutinins and autoagglutinins. Of importance here is that only 3 out of the 28 patients with positive reactions had never been transfused or been pregnant. The significance of previous pregnancy or transfusions will be discussed presently. In 144 cases of nonthrombocytopenic controls, only 16 displayed agglutinins of any kind, but only 2 of these 16 had never been transfused nor pregnant. In 75 patients of the nonthrombopenic control group, there was not a single instance in which autoagglutinins were found. The patients within these control groups with positive agglutinins are considered chiefly as examples of isoimmunization, the isoagglutination occurring mainly as the result of multiple transfusions.

Harrington et al.[356] have reported 8 types of platelets; others[357] recognize 4 groups of serologically distinct platelet groups. These various platelet types have antigenicity in the same way as red cell types, and transfusions of mismatched platelets will result in antibody formation. Apparently, isoagglutinins for platelets can also be induced during pregnancy in a manner similar to that responsible for Rh incompatibility between the mother and the fetus.

The finding of autoantibodies in the blood of patients with thrombocytopenic purpura poses a number of questions. Clinicians have constantly been in search of criteria for splenectomy in these patients, and serologic tests of the type being discussed here were immediately looked upon as possible aids in this direction. Thirty-eight out of the 50 patients with positive agglutinins were splenectomized. Twenty-five of these had a complete remission, 4 patients had a partial remission, and the remaining 9 were not benefited by surgery. In the group of 22 patients without demonstrable agglutinins, 16 were splenectomized with the following results: 2 complete remission, 4 partial remission, and 10 not benefited. Although these figures indicate that the prognosis with splenectomy is apparently better when agglutinins are demonstrable, Harrington cautions that "more experience is needed, however, before it would be permissible to state with certainty that the presence or absence of platelet agglutinins should be a major criterion for the selection of candidates for splenectomy."*

Autoantibodies in a mother may result in neonatal thrombocytopenia in infants; Harrington cites 9 such cases. This may occur even though a remission has been induced in the mother by splenectomy. Harrington questions the utility of platelet transfusions in patients showing autoantibodies since platelets disappear readily from the circulation of such patients. He would reserve such measures only as temporizing in patients presenting serious hemorrhage intracranially or within the gastrointestinal tract. Harrington warns against the use of patients with a history of thrombocytopenic purpura as blood donors since antibodies may persist for years even though remission has been induced by splenectomy.

Isoimmunization may create problems akin to autoimmunization. Since multiple transfusions may induce isoimmunization, individuals receiving such transfusions should be screened as future donors of whole blood or plasma. Isoimmunization may occur in pregnancy, as previously noted. Harrington suggests: "Rejection of all candidates (for blood donors) who have received more than 20 transfusions of whole blood or anyone who has been transfused or pregnant within one year would seem to be reasonable minimum precautions at the present time."* Harrington points out the lack of feasibility of protracted platelet transfusions in patients with thrombocytopenic purpura especially of the secondary type; these patients develop isoimmunization and even refractoriness after 3 to 6 such transfusions and further pursuit of such therapy is valueless.

A third problem related to isoimmunization is primary neonatal isoimmunization occurring in infants born to mothers who have not had thrombocytopenic purpura. Isoimmunization

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*From Harrington, W., et al.: Ann. Int. Med. **38:** 433, 1953.

in the mother may have come about through previous transfusions, pregnancy, or both. Such isoimmunizations correspond to that seen with Rh incompatibility in that the mother displays no symptoms.

As technics improve, agglutinin detection in the thrombopenic states will improve. Adelson and Stefanini[358] describe a heterologous (Forssman) type of platelet agglutinin in the case of thrombotic thrombocytopenic purpura. Phase contrast microscopy studies[359] of the bone marrow of normal recipients of plasma from a patient with a high-titer platelet agglutinin revealed changes in the megakaryocytes of the recipients similar to those seen in idiopathic thrombocytopenic purpura. These observations suggest that the antiplatelet factor acts by (1) attacking platelets being discharged from megakaryocytes, (2) preventing normal platelet formation from megakaryocytes, and (3) causing maturation arrest of megakaryocytes.

Laboratory methods in investigation of thrombocytopenic purpura

Platelet count

The platelet count is described in the discussion of platelets.

*Platelet agglutination test**

Reagents:

1. Silicone.
2. Ethylene diamine tetraacetic acid, disodium salt, 5%, in distilled water.

Materials:

1. 18-gauge needle.
2. 16 × 100 mm. test tubes.
3. 10 × 75 mm. test tubes.
4. Parafilm.
5. Broad-bore 1 ml. pipets graduated in tenths.
6. 37° C. water bath, 56° C. water bath.
7. Mechanical serologic rotating platform.
8. Depressed cell slides.

All glassware that comes into contact with platelets is siliconized. Needles are not coated.

Procedure:

1. Add 10 ml. blood for platelet suspensions to 0.4 ml. EDTA. Obtain blood from donors compatible with the test sera. It is good practice to use Group O donors whose platelets do not agglutinate readily. Some normal donors have platelets which require much care to prevent agglutination by their own sera. Allow the blood to flow directly into a 16 mm. tube after the first few ml. have escaped into a gauze flat under the hub of the needle. The venipuncture must be accomplished cleanly, and only moderate tourniquet pressure must be used.
2. Cap the tube with Parafilm, invert a few times to ensure adequate mixing of the EDTA and blood, and then centrifuge at 150 g for 20 min. The supernatant platelet-rich plasma constitutes the platelet suspension and should contain 300,000-400,000 platelets/c.mm. on direct count. If the count is higher, a smaller vol. should be employed. If the count is lower but over 100,000/c.mm., the suspension can be used.
3. Obtain sterile test serum by incubating clotted blood in an uncoated test tube for 24 hr. at 37° C. Then separate the serum, using sterile technic, and incubate at 56° C. for 45 min.
4. Add 0.1 ml. EDTA to each 2 ml. serum.
5. Place 0.1 ml. platelet suspension in each 10 × 75 mm. test tube and add 0.4 ml. treated test serum.
6. Shake the tubes to accomplish mixing and place in 5° C. refrigerator for 12 hr.
7. Shake gently and pour ½ their contents into the depressed cell slides.
8. Agitate at 100 rotations/min. for 5 min.
9. Read microscopically at 100×. Wide field oculars facilitate the reading.
10. Platelet clumping is reported as positive or negative.

Laboratory findings in thrombocytopathias (including thrombocytopenia)

Bleeding time: Prolonged.
Tourniquet test (Rumpel-Leede): Positive.
Coagulation time: Prolonged.
Clot retraction: Poor.
Platelet count: Low to normal.
Prothrombin consumption: Abnormal.
Platelet agglutins: May be present.

Thrombotic thrombocytopenic purpura[366, 367]

This is a rather rare febrile disease which pathologically is characterized by arterial and capillary platelet thrombi. The hematologic consequence is severe thrombocytopenic purpura which is accompanied by a hemolytic anemia of unknown etiology. Apart from the purpura and anemia, bizarre neurologic manifestations dominate the clinical picture. The etiology of the disease is not yet known, but some features point to primary vascular disease involving the ground substance. Many of the plugged vessels show deposits of subendothelial fibrin or fibrinoid material. The thrombi contain not only platelets but also fibrin.

The historical review of this disease which is still at times called Moschcowitz's disease is rather interesting. Moschcowitz[368] noted a case which he called "an acute febrile pleiochromic anemia with hyaline thrombosis of the terminal arterioles and capillaries." He called it an undescribed disease although its essential characteristics definitely suggest its similarity to the case of acute hemolytic anemia described by Lederer. In his patient, a 16-year-old girl, the onset was sudden with a rise in temperature, a few petechiae on the arm, and a red blood count of 1,330,000, hemoglobin of 40%, leukocytes 12,600, of which 65% were polymorphonuclear cells. A fragility test showed hemolysis complete at 0.19%. Urine showed albumin and hyaline and granular casts. The patient showed partial paralysis of the left arm and leg and a slight facial paralysis and died 14 days after the onset of the disease. A partial necropsy was done. The heart muscle showed thrombi in the terminal arterioles or capillaries. Vessels were plugged with hyaline masses. These thrombi were first described by Klebs,[369] who noted them in extensive burns. Schmorl[370] found them in the stomach and liver after abrine poisoning. Flexner[371] showed that they arose from agglutinated red cells, proving this by injecting the highly agglutinative substance, ricin, into rabbits.

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*Courtesy W. J. Harrington, Washington University, St. Louis, Mo.

Epidemic hemorrhagic fever

In June, 1951, United Nations Troops in Korea encountered an acute disease characterized by fever, prostration, vomiting, a variety of hemorrhagic manifestations, shock, proteinuria, and renal failure. A disease known to the Japanese as epidemic hemorrhagic fever and to the Russians as epidemic hemorrhagic nephroso-nephritis or Far Eastern hemorrhagic fever has been described in 2 United States Army Technical Bulletins on Korea and Manchuria.[372, 373]

This disease was first described by the Russians who encountered it in Far Eastern Siberia in the middle 1930's. They found that the incubation period was generally 10-15 days, that a single attack conferred immunity, and that the disease-producing agent would pass through a size N Berkefeld filter. It has been impossible to reproduce this disease in animals. In spite of the failure of animal experiments, it is suggested that hemorrhagic fever may be transmitted to man by rodent ectoparasites, such as fleas, mites, ticks, and chiggers. These chiggers seem best to fit the observed epidemiologic facts.

The complete story on this interesting and new disease is found in the *Symposium on Epidemic Hemorrhagic Fever* by various authors.[374] Briefly, the clinical picture is characterized by sudden onset, in a person recently situated in an endemic area, of chills, fever, prostration, frontal headache, marked thirst, and myalgias. There is a marked facial flush, injection of the palate and conjunctivae, and petechiae of the conjunctivae, palate, axillary folds, and waistline. Proteinuria and reduction in specific gravity of the urine are present.

This clinical course may be divided into 4 phases, each designated by a characteristic physiologic aberration: (1) febrile, (2) hypotensive, (3) oliguric, and (4) diuretic.

In this Symposium, Lt. Robert J. Lukes describes the pathology of 39 fatal cases of this disease. There was almost constant occurrence of hemorrhage in the right atrium of the heart, of severe congestion, hemorrhage, and infarctlike necrosis in the renal medulla, and of hemorrhage and necrosis in the anterior lobe of the pituitary gland, in association with the pattern of the vascular changes. All this supports the contention proposed in the Japanese literature and by Steer and Hullinghorst that capillary damage is the basic process. Retroperitoneal edema, observed only once in secondary shock, is the striking pathologic feature when the patient dies during shock in early phases of the disease. The abrupt increase in incidence of pulmonary edema in the oliguric phase, particularly after the eighth day of the disease when retroperitoneal edema is rare, supports the clinically suspected role of redistribution of fluid and electrolytes with resorption of the abnormal interstitial fluid.

The frequent coexistence of pulmonary edema and bronchopneumonia in the oliguric and diuretic groups and the high incidence of pulmonary abscess formation support the concept of Moon that pulmonary edema of the albuminous type is conducive to the development of pulmonary infections.

The presence of necrosis of the anterior lobe of the pituitary gland in cases suggests that shock is not the only major factor in the pathogenesis of pituitary necrosis in hemorrhagic disease. Anoxemia is also an important factor and is probably the major cause of necrosis in the absence of shock.

Studies of the coagulation mechanism in epidemic hemorrhagic fever reveal abnormalities in prothrombin activity, clotting time, and platelet count. Persistence of thrombocytopenia, which develops early in the disease, through the oliguric-hypertension phase would appear to be causatively related to the bleeding phenomenon observed. Occasional marked deficiencies in plasma coagulation factors are found in seriously ill patients and may contribute to clinically significant bleeding. This plasma coagulation defect is similar to that observed in acute renal insufficiency and has not been clearly defined.

Studies by Giles and Langdon in this report suggest that some mechanism other than decrease in total blood volume is a primary cause of shock in hemorrhagic fever. Although most patients showed normal red cell volumes during the febrile and shock phases of the disease, two showed a significant decrease during severe shock, and many others have decreases in other phases.

Studies by Froeb and McDowell in this report in the *American Journal of Medicine* on renal function in hemorrhagic fever show that the ability to form urine of high specific gravity may persist after the initial decrease in C_{IN} (inulin clearance) and C_{PAH} (*p*-aminohippuric acid clearance) but is subsequently lost. The clearance returns to normal before the concentrating ability.

The following electrolyte abnormalities were observed during the course of hemorrhagic fever. (1) During the oliguric phase, hyponatremia, hyperkalemia, hypocalcemia, and retention of anions were common. However, serious hyperkalemia was rarely encountered, and acidosis was not severe. (2) During diuresis, hyponatremia occasionally resulted from a salt-losing necropathy but more often was due to unknown causes. The hyperkalemia frequently increased during the early diuresis but at this time was not dangerous. Potassium deficiency, associated with the usual symptoms and signs of hypokalemia, sometimes occurred even though hypokalemia was not present. Hunter thus describes electrolyte abnormalities in hemorrhagic fever.

HEMORRHAGIC DIATHESIS DUE TO DEFECTS IN COAGULATION FACTORS

These defects may be hereditary and congenital or acquired.

Afibrinogenemia and hypofibrinogenemia[376-380]

There are 2 types of afibrinogenemia and hypofibrinogenemia—congenital and acquired.

Congenital afibrinogenemia

The rare congenital form of afibrinogenemia is associated with severe hemorrhages that involve the skin and mucous membranes, leading at times to uncontrollable bleeding following relatively minimal trauma (needle stick). The disease is transmitted as a mendelian recessive character and is not sex-linked. It is frequently associated with thrombocytopenia.

Acquired hypofibrinogenemia

Acquired hypofibrinogenemia is seen in liver diseases and maladies involving the reticuloendothelial system. The loss of fibrin can be explained on the basis of intravascular defibrination by the formation of multiple small clots or by the abnormal activation of the fibrinolytic system. Hypofibrinogenemia occurs following operation on the lungs and prostate but is most important clinically as a life-endangering complication of obstetrical conditions such as postpartum hemorrhage, retained secundinae, hemorrhage associated with premature separation of the placenta, and hemorrhage accompanying intrauterine fetal death (dead fetus syndrome). The escape of amniotic fluid, rich in thromboplastin, into the general circulation may lead to the formation of microthrombi in various organs and thus to hypofibrinogenemia. On the other hand, the hypofibrinogenemia may be entirely due to activation of the fibrinolytic system since placenta and uterus contain tissue activators which convert plasminogen (profibrinolysin) to plasmin (fibrinolysin).

Laboratory diagnosis

Screening procedures

If the blood sample in the test tube fails to clot within 6 min. or if an abortive clot forms and lyses rapidly within 1 hr., the presence of hypofibrinogenemia must be suspected. Hemophilia, rare in women, is the only other condition in which blood fails to clot.

Several rapid test methods such as Fibrindex* and Fi-Test† are available commercially.

Quantitative method—estimation of plasma fibrinogen—Goodwin[375]

Principle: Fibrinogen in plasma is precipitated by the addition of sodium sulfite. The precipitate is washed with sodium sulfite and then dissolved in sodium phosphate by heating. Finally, the biuret reaction for proteins is carried out by the addition of a solution of copper sulfate to the dissolved protein. The density of this colored solution is then measured spectrophotometrically.

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*Ortho Pharmaceutical Co., Raritan, N. J.

†Hyland Laboratories, Los Angeles, Calif.

Reagents:

1. Sodium sulfite, 13%.
    (a) Dissolve 130 gm. sodium sulfite in approximately 800 ml. warm water, transfer to a volumetric flask, and dilute to 1 L.
2. Trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$), 5% aqueous solution.
3. Cupric sulfate ($CuSO_4 \cdot 5H_2O$), 4% aqueous solution.

4. Protein standards. Ideally the protein used for preparation of standards should be human fibrinogen; however, its availability is limited, and as supplied, the salt content of the dried product is not defined so that a weighed amount of this product will contain an undefined amount of fibrinogen. Since this is true, alternative methods of preparing standards will be described; the choice of method should be dictated by the resources of the individual laboratory.
    (a) Fibrinogen standards. Human fibrinogen is available commercially.*
        (1) Stock standard. Dissolve 1.25 gm. fibrinogen in 100 ml. 0.85% sodium chloride. Determine the protein concentration by Kjeldahl analysis† (p. 41) and adjust the concentration to exactly 1 gm./100 ml. by the addition of required vol. 0.85% sodium chloride solution.

Note: In order to convert fibrinogen quantitatively to ammonia it is necessary to use a catalyst and a longer digestion than that required for most serum proteins.

    (b) Albumin standards.
        (1) Stock standard. Prepare a solution of crystalline bovine albumin (available from the same source as fibrinogen) to contain approximately 1 gm./100 ml. Determine the protein concentration by the method used in your laboratory. Adjust the concentration to exactly 1 gm./100 ml. A concentrated solution of human instead of bovine albumin may be diluted if desired.
    (c) Working standards. Dilute 1, 2, 3, 4, 5, 6, 7, and 8 ml. stock standard solution each to a total volume of 10 ml. with 0.85% sodium chloride. These standards are equivalent, respectively, to 0.10, 0.20, 0.30, 0.40, 0.50, 0.60, 0.70, and 0.80 gm.% fibrinogen when used as directed in the calibration procedure.

Sample: The sample to be used is plasma obtained from either oxalated or heparinized blood. Hemolysis should be minimized.

Procedure:

1. Pipet 1 ml. plasma into a 12 ml. graduated centrifuge tube.
2. Add 9 ml. 13% sodium sulfite solution, mix thoroughly, and incubate in a water bath at 37° C. for 10 min.
3. Centrifuge at 2500-3000 r.p.m. for 10 min. Pour off the supernatant fluid, drain the tube, and wipe the lip of the tube.
4. Break up the precipitate by blowing in forcibly 5 ml. sodium sulfite solution.
5. Cap the tube and shake vigorously to suspend the precipitate.
6. Wash down the walls of the tube with an additional 3 ml. sodium sulfite.
7. Recentrifuge, pour off the supernatant fluid, and drain the tube.
8. Add 8 ml. sodium triphosphate solution to the precipitate in the tube and heat in a boiling water bath until the precipitate dissolves. This usually requires 10-15 min.
9. Cool the tube and adjust the volume of solution in the tube again to 8 ml. by the addition of distilled water and mix thoroughly.
10. Add 0.2 ml. copper sulfate solution to the tube, stopper, and shake vigorously for 2-3 min.
11. Centrifuge at 2500-3000 r.p.m. for 10 min.
12. Decant the supernatant fluid into a cuvet.
13. Determine the % T of this solution at 560 mμ, using a **blank** composed of a mixture of 8 ml. sodium phosphate solution and 0.2 ml. copper sulfate which has been shaken and centrifuged, as has the unknown.
14. Refer this value to the calibration table to obtain gm.% fibrinogen.

Calibration procedure:

1. Pipet 1 ml. each of the working standards into centrifuge tubes.
2. Add 7 ml. sodium phosphate solution to each tube and mix thoroughly.
3. Add 0.2 ml. copper sulfate solution to each, shake, and centrifuge as for the unknowns.
4. Pour the supernatant solutions into cuvets and determine the transmittance of each as described in the procedure for unknowns.
5. Prepare a concentration-transmittance chart from the values obtained.

Discussion: This method compares favorably with the more tedious methods which make use of thrombin precipitation and gives more reproducible results than those obtained with the turbidimetric procedures.

The volume in which the final color reaction is carried out may be modified to suit the particular photometer used, but when modified, the ratio of the vol. copper sulfate solution to protein solution should always be the same as described here.

Fibrinogen test—Andersch-Gibson modification[381]

Reagents:

1. Sodium chloride solution, 0.9%.
    (a) Dissolve 9 gm. sodium chloride in distilled water and dilute to 1000 ml. with distilled water.
2. Calcium chloride, 0.25%.
    (a) Dissolve 250 mg. calcium chloride in distilled water in a 100 ml. volumetric flask and dilute to 100 ml. with distilled water.
3. Sodium hydroxide, 10%.
    (a) Use 10 gm. NaOH/100 ml. total vol.

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*Pentex Laboratories, Inc., Kankakee, Ill.

†Fibrinogen contains 16.9% nitrogen.

4. Sodium hydroxide, 20%.
   (a) Use 20 gm. NaOH instead of 10 gm.
5. Standard tyrosin solution.
   (a) Add 250 mg. tyrosin to about 50 ml. N/10 hydrochloric acid in a 100 ml. volumetric flask and dilute to 100 ml. with N/10 HCl.
6. Phenol reagent of Folin and Ciocalteu (p. 46).

Procedure:

1. Place 25 ml. 0.9% sodium chloride solution in a test tube and add 1 ml. plasma.
2. Mix and add 2 ml. 0.25% calcium chloride, placing a small stirring rod in the mixture and allow it to stand for 1 hr.
3. Remove stirring rod containing fibrin and wash with distilled water.

Unknown:

1. Place stirring rod in small tube and add 0.5 ml. 10% sodium hydroxide.
2. Place in boiling water for 30 min.
3. Cool and add 7.5 ml. distilled water.

Standard:

1. Place 2 ml. standard tyrosin solution in a large test tube and add 5.5 ml. distilled water and 0.5 ml. 10% sodium hydroxide.
2. Add to both unknown and standard 1 ml. phenol reagent and 3 ml. 20% sodium hydroxide solution; mix and read in a colorimeter after 30 min.

Calculation:

10/R × 100 × 0.5 mg. × 13 = mg. fibrinogen in 100 ml. plasma.

Range of fibrinogen is 200-400 mg./100 ml. plasma.

Hypoprothrombinemia[382]

There are 2 types of hypoprothrombinemia—congenital and acquired.

Congenital hypoprothrombinemia

Three types of hereditary bleeding diseases exist which have in common a markedly prolonged Quick 1-stage prothrombin time: (1) true hypoprothrombinemia, (2) factor V deficiency, and (3) factor VII deficiency. These diseases are transmitted as nonsex-linked recessive traits.

Acquired hypoprothrombinemia

Acquired hypoprothrombinemia follows vitamin K deficiency, parenchymal liver disease, and therapy with anticoagulants.

1. Vitamin K deficiency. This deficiency is due to difficulty in absorption of vitamin K rather than to inadequate intake. Vitamin K deficiency may be produced by colitis (diarrhea), suppression of coliform bacteria by antibiotics, the abacterial state of the colon of newborn infants, or obstructive jaundice. The various malabsorption syndromes are also included in this classification.
2. Parenchymal liver disease. The inability of the parenchyma of the liver to utilize vitamin K for the production of prothrombin leads to a lowering of the blood level of prothrombin.
3. Therapy with anticoagulants.

Laboratory diagnosis

Prolongation of the Quick 1-stage prothrombin time occurs. If necessary, correction studies can be employed to identify the defect. Stored serum should correct factor VII deficiency. Fresh barium sulfate plasma should correct factor V deficiency, and stored oxalate plasma should correct prothrombin deficiency.

Factor V deficiency

There are 2 types of factor V deficiency: congenital and acquired.

The congenital form as described by Owren[383] is a hemophilia-like disease associated with internal hemorrhage but does not have pronounced joint manifestations.

The acquired form appears in liver disease in conjunction with several other coagulation defects. It has also been described in purpura fulminans and in diseases involving the reticuloendothelial system.

Factor VII deficiency

Factor VII deficiencies may be either congenital or acquired.

The congenital form was described by Owren. Only slight bleeding tendencies are present.

The acquired form is found in all the conditions which lead to defects in prothrombin and factor V.

Hemophilias

Hemophilias are hereditary diseases transmitted as sex-linked recessive characteristics leading to defects which remain the same from generation to generation. Clinically the most constant finding is hemarthrosis, but hemorrhages may occur in many other locations such as the retroperitoneum, pharynx, etc. Certain age periods are particularly dangerous, such as the infant stage (circumcision), the toddler state (dentition and injuries), and puberty. In later life the hemorrhages diminish.

Hemophilia A

Hemophilia A is the classic hemophilia due to a defect in antihemophilic globulin (factor VIII) which may vary from almost complete absence (less than 1%) to a moderate or only slight diminution, leading to various degrees of severity of the disease. The defect lies in the thromboplastin generation and is genetically determined by a gene and its alleles. Hemophilia A is 3-4 times more common than hemophilia B.

Hemophilia B (Christmas disease)

On the basis of the thromboplastin generation test, it is possible to differentiate hemophilia B from the classic type, hemophilia A. Hemophilia B is due to a factor IX deficiency which interferes with the thromboplastin generation and produces a clinical picture which cannot be differentiated from that of hemophilia A. Genetically it is determined by its own gene and does not represent an allelic form of hemophilia A.

Comments: Not too many years ago, most of us had the impression that we fairly well knew the classic disease hemophilia as an entity occurring only in males and transmitted through females as a recessive sex-linked mendelian characteristic. The abnormal bleeding tendency has been known from the early days of the Hebrews who recognized its occurrence after circumcisions, and more recent history has been enlivened by the occurrence of hemophilia in the royal families of Europe. Within medical circles, the abnormally prolonged clotting time of hemophilic blood was well known, and the therapeutic value of blood transfusions was well established.

Any complacency about our knowledge of hemophilia has been fervently shaken by the coagulationists. This has come about chiefly through recent advances in knowledge of the factors involved in blood coagulation. Hemophilia is no longer a simple disease, and there is some question, even in the minds of experts, as to just what the disease is. Dameshek[384] has collected the views of outstanding experts in the field in an attempt to answer this question.[385-393]

As might be expected, there are the same differences of opinion about defining hemophilia as there are concerning nomenclature and theories of blood coagulation. The center of the controversy is the nature of the plasma and platelet factors, excluding calcium, which take part in the conversion of prothrombin to thrombin. A brief glance at the theories of blood coagulation in this area readily reveals many but not all of the differences. We heartily recommend reading this symposium of Dameshek as an enlightening (or perplexing) presentation of how utterly stirred up the concept of hemophilia has become.

Whereas in times past our clinical concern about hemophiliacs was limited to males, we now must consider both male and female bleeders as possible hemophiliacs. Hereditary patterns are being found in the various newly described forms of hemophilia or hemophilia-like diseases as was true of the classic disease. However, acquired defects of clot-promoting factors have been recognized in syndromes simulating hemophilia.

The laboratory diagnosis of hemophilia traditionally rested on a prolonged clotting time. This is no longer true. Although most of the hemophilia-like diseases may have a prolonged clotting time, many instances with a normal coagulation time are being uncovered. Today, in the study of any patient with a bleeding diathesis **not otherwise explained,** one must investigate a plasma defect or defects as possible causes of the disease. There are many tests of varying complexity which can help delineate these defects. Some of these have been elaborated in the section on technic and others will be mentioned here briefly in the following outline:

1. The Quick prothrombin consumption time or the serum prothrombin time. Hemophiliacs show a very short (±10 sec.) time. Hemophilia-like states may show a **shortened** or almost **normal time.** This test is nonspecific insofar as the plasma factors are concerned.
2. Standard prothrombin activity tests, e.g., Quick 1-stage or modifications thereof. These tests show normal times in classic hemophilia but may be abnormal in some of the hemophilia-like diseases.
3. Owren technics for specific factors, such as prothrombin, proconvertin, and proaccelerin. These tests demonstrate specific defects in hemophilia or hemophilia-like disease. It was by the use of such technics that Owren discovered parahemophilia (or factor V) deficiency.
4. The thromboplastin generation test.[394] This test is helpful in distinguishing and assaying various components of the thromboplastin-producing system.
5. The antihemophilic globulin (AHG) assay[280] test measures the specific defect in hemophilia and is useful in detecting latent subclinical cases. Normal AHG assays at 100%, whereas classic hemophilia assays at 0%, moderate hemophilia less than 3%, mild hemophilia 16%, and subhemophilia 33%.
6. The partial thromboplastin test (PTT).

This is a screening procedure in which the plasma of patients with hemophilia and hemophilia-like disease may be brought under scrutiny. The authors[280] have hypothesized the following diagnostic groups: hemophilia, hemophilioid state A, hemophilioid state B, hemophilioid state C, and hemophilioid state D. In all of these the routine PTT gives a prolonged time. However, if equal parts of fresh normal barium sulfate-treated plasma are added to the test plasma, the results aid in differentiation of the states. With such fortified plasmas in hemophilia and hemophilioid state A the partial thromboplastin time returns to normal, but in hemophilioid states B, C, or D the partial thromboplastin time remains prolonged.

Table 244

	Prothrombin time	
	Untreated plasma	*Fortified plasma*
Hemophilia	Normal	Normal
Hemophilioid state A	Prolonged	Normal
Hemophilioid state B	Prolonged	Prolonged
Hemophilioid state C or D	Normal	Normal

These authors have further elaborated their detection and separation system by making standard prothrombin time determinations on untreated plasma and fortified plasma (Table 244).

7. Matching experiments. The basic principle of such tests is determining whether mixing the blood of one individual with that of another corrects a known defect. Such matching experiments originally aided in delineating true hemophilia from some of the subvarieties. It was found that in these subgroups the patient's blood would correct the defect in hemophilia in the same manner as normal blood. Since then, this procedure has become standard practice in the study of hemophilia and related disorders. This technic is limited to separating the entities but does not delineate the specific deficiency. However, an understanding of such matching experiments is helpful in determining therapy. As an example, classic hemophilia will respond to con-

Table 245. *Summary of clinical and clotting characteristics of AHG, PTC, and PTA deficiencies**

	Antihemophilic globulin (AHG) deficiency	*Plasma thromboplastin component (PTC) deficiency*	*Plasma thromboplastin antecedent (PTA) deficiency*
Sex	Male (female very rarely)	Male	Male and female
Hereditary pattern	Sex-linked, recessive trait carried by female and transmitted to male	Similar to AHG deficiency	Not sex-linked; transmitted to male and female by female and probably male carriers
Pattern of hemorrhage:	Depends upon clotting time and the degree of the clotting defect:	Depends upon clotting time and the degree of the clotting defect:	Depends upon clotting time and the degree of the clotting defect:
Degree	Slight to very severe	Slight to moderately severe	Slight to moderate
Sites	Joints frequent, follows trauma and any operative procedure	Joints sometimes involved; follows trauma and operative procedures	Joints rarely involved; most usually follows minor surgical procedures as tonsillectomy, tooth extraction, etc.
Clotting time	Normal to over 1 hr.	Normal to about 60 min.	Normal to 30 min.
Clotting defect corrected by:			
Normal plasma	Yes	Yes	Yes
Normal $BaSO_4$-treated plasma	Yes	No	Yes
Normal serum	No	Yes	Yes
Fraction I	Yes	No	No
Normal $BaSO_4$-treated serum	No	No	Yes
Other findings	Normal values—platelet count, prothrombin time, clot retraction Usually normal—bleeding time, tourniquet test Abnormal (degree related to the extent of the deficiency)—heparin clotting time, serum prothrombin time, recalcified clotting time of slowly and rapidly centrifuged plasma, thromboplastin generation		

*From Rosenthal, R. L.: Am. J. Med. 17:57, 1954.

centrated antihemophilic globulin administration, but other coagulation factor deficiency diseases will not respond favorably.

A typical series of such matching experiments may be found in the paper by Brinkhous et al.[280]

One of the difficulties in matching experiments has been the need for blood from a patient with the deficiency of plasma factor. As the various factors become elaborated, their reproduction in the test tube is becoming more frequent, making such experiments available to more observers. Ideally, the isolation and purification of factors by physical and chemical means would help considerably. Such isolation is understandably difficult when one considers the complexity of the coagulation mechanism.

As a result of studies such as those mentioned have come such names as Christmas disease,[395] parahemophilia,[396] hemophilioid states A, B, C, and D,[280] PTC,[397] plasma thromboplastin antecedent (PTA) deficiency.[398] In addition, there have been designations of various factor deficiencies by number, such as factor V, VI, or VII deficiencies. There is no unanimity of opinion as to which system of nomenclature to adopt, and often there is some question as to whether authors using different terminology are discussing the same syndrome.

Rosenthal[399] presents an enlightening article on the clinical, laboratory, and hereditary patterns of this group of diseases. Table 245 presents an excellent bird's-eye view of plasma factor deficiencies.

Whatever else may be said about these new entities, there is no question that this elaboration has added considerably to knowledge related to the coagulation mechanism.

Laboratory diagnosis of hemophilias

Coagulation time: Prolonged (but results depend on severity of disease).

Bleeding time: Normal.

Prothrombin time: Normal.

Prothrombin utilization: Deficient.

Thromboplastin generation test: Deficient but can be corrected by fresh plasma or blood.

The diagnosis of the hemophilia trait is a difficult one, suggested at times by slight deficiencies in the prothrombin utilization, in prothrombin consumption, and in factor VIII.

PTA deficiency and Hageman defect

These are hemophilia-like (hemophilioid) conditions which are hereditary but autosomal and can affect either males or females. Both represent a defect in the thromboplastin synthesis.

COAGULATION DEFECTS DUE TO CIRCULATING ANTIBODIES

Hyperheparinemia

Hyperheparinemia may be either congenital or acquired.

Congenital hyperheparinemia

Quick described one patient who had no epistaxis, hemarthrosis, or petechiae but bled profusely following injury (delivery). The amount of circulating heparin was sufficient to prevent any thrombin formation.

Acquired hyperheparinemia

Thrombin inhibitors appear in a great variety of conditions such as shock, allergies, collagen disease, thermal and radiation burns, liver diseases, leukemias, and malignant tumors.

Tocantins[400, 401] described an antithromboplastic substance (anticephalin) which in some cases of hemophilia A exceeds in functional activity the antihemophilic globulin which is present in normal quantities.

TRANSFUSION THERAPY OF COAGULATION DEFECTS

Since the laboratory may be responsible for providing the necessary blood factors for a bleeding patient or at least for procuring them, a short list of the defects and their correction by blood or blood fractions follows.

Defect	*Correction factor*
Acute blood loss	Stored blood, fresh blood, plasma, Dextran
Afibrinogenemia	Fresh blood, fibrinogen, lyophilized plasma, stored blood (fresh)
Agammaglobulinemia	Fresh blood, gamma globulin, stored blood
Dicumarol bleeding	Stored blood (fresh), vitamin K_1, fresh blood
Factor V deficiency	Fresh blood, AHG
Factor VII deficiency	Fresh blood, type-specific frozen plasma, lyophilized plasma, stored blood (fresh)
Fibrinolysins	Fresh blood, fibrinogen, lyophilized plasma, stored blood (fresh)
Hemophilia A	AHG, fresh blood, frozen plasma, lyophilized plasma
Hemophilia B	Fresh blood, AHG, frozen plasma, lyophilized plasma
Heparin bleeding	Protamine sulfate

Defect—cont'd	*Correction factor*—cont'd
Hypofibrinogenemia	Fresh blood, fibrinogen, lyophilized plasma, stored blood (fresh)
Hypoprothrombinemia	Fresh blood, stored blood (fresh), lyophilized plasma
Liver diseases	Fresh blood, frozen plasma, albumin
Thrombocytopenia	Platelet concentrates, fresh blood

Chapter 64

The anemias

JOHN D. BAUER

The term anemia is a clinical concept which was used originally to designate a decrease in the total quantity of blood. It is now applied to diseases accompanied by a decrease in the number of erythrocytes or hemoglobin content or both. A clinically important point is that the decrease must be greater than 10% to be of significance. Symptomatically and physiologically, the important factor is the relationship of tissue oxygen needs to the oxygen supplied by the blood.

CLASSIFICATION

There is no one single type of classification that serves all purposes, i.e., diagnosis, prognosis, treatment, and pathophysiology.

Wintrobe[402] grouped the anemias on the basis of the absolute constants of the red cells and hemoglobin.

Classification by mean corpuscular volume (MCV)

- Microcytic anemia—MCV less than $80\mu^3$
  - Etiology
    - Iron deficiency
    - Thalassemia
    - Pyridoxine deficiency
    - Hemoglobin variants
    - Infection
    - Lead poisoning
- Normocytic anemia—MCV equal to $80\text{-}95\mu^3$
  - Etiology
    - Hemolytic anemias
    - Blood loss (up to 3 mo.)
    - Aplastic or hypoplastic anemia
    - Myelophthisic anemia
    - Anemias of pregnancy
    - Anemia of infection
    - Anemia of uremia (kidney diseases)
    - Anemia due to endocrine disturbances
- Macrocytic anemia—MCV greater than $95\mu^3$
  - Etiology
    - Vitamin B_{12} deficiency
    - Folic acid deficiency
    - Anatomical alterations of small intestines
    - Liver disease
    - Hypothyroidism
    - Pregnancy
    - Interference with nucleic acid synthesis (thymine, vitamin C deficiencies, etc.)
    - Hemolytic anemia with early release of marrow reticulocytes
- Diphasic anemia—combination of microcytic and macrocytic

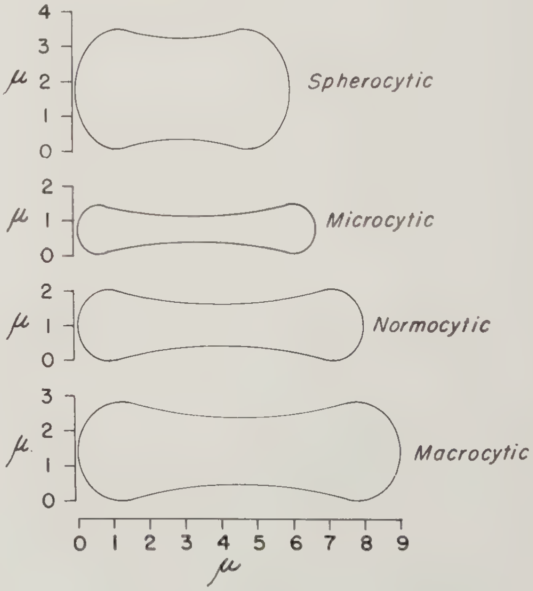


Fig. 405. Schematic representation of side views of various types of red blood cells.

Etiologic classification

This classification presents another approach to the aforementioned morphologic classification based on MCV.

- Interference with red cell production
  - Deficient erythropoietic substances
    - Iron deficiency
    - B_{12} deficiency
    - Folic acid deficiency
    - Pyridoxine deficiency
    - Vitamin C deficiency
    - Magnesium deficiency (?)
    - Thiamine deficiency
    - Orotic acid deficiency
  - Deficient erythropoietin
    - Uremia
    - Infection
    - Endocrine disturbances
  - Marrow replacement
    - Atrophy (drugs)
    - Tumor invasion
    - Fibrous or osseous hyperplasia
  - Dysfunction of marrow
    - Thalassemia
    - Aplastic anemia (interference with release of cells)
- Loss of red blood cells
  - Blood loss (hemorrhage)
  - Hemolysis
    - Intrinsic red cell defects (e.g., spherocytic anemia, hemoglobinopathies, and enzymatic defects)
    - Extrinsic defects (drugs, autoantibodies)
    - Symptomatic hemolytic anemias (lupus, lymphomas, etc.)
    - Hyposplenism

Erythrokinetic classification

Before this classification is given, terminology and methods of investigation of erythrokinetics will be discussed briefly.

Erythrokinetics

This term refers to the quantitative measurement of production and destruction of red cells and their relationship to each other. Normally, production and destruction are in equilibrium. In some anemias this balance is maintained, whereas in others it is lost.

To appreciate erythrokinetics, tests are employed which measure red cell production and red cell destruction.

Terminology:

Total erythropoiesis: Total red cell production irrespective of whether the cells enter the peripheral circulation or not.

Effective erythropoiesis: The cells that enter the circulation and remain there for at least 24 hr.

Ineffective erythropoiesis: The defective cells that remain in the bone marrow and never enter the circulation but are absorbed and destroyed within the marrow.

Methods of investigation of erythrokinetics

Production of red blood cells

Reticulocyte count (measure of effective erythropoiesis): This is considered in the discussion of reticulocytes. These are young red blood cells which have just entered the circulation, are larger than mature red cells, and contain a fine reticulum of ribonucleic acid which disappears in about 24 hr. The reticulocyte response reflects the marrow production of new red blood cells and their release into the circulation. If the reticulocyte response is expressed as percentage of circulating mature cells, 2 factors will determine the percentage: the total number of mature cells and the number of reticulocytes. If the first is decreased, an increase of the latter will result which does not truly reflect bone marrow activity. On the other hand if the reticulocyte count is expressed as reticulocytes/c.mm. whole blood (normal 60,000/c.mm.), the count becomes meaningful and related to erythropoiesis. In megaloblastic anemias in which the erythroid marrow is extremely hyperplastic, the paradox exists that the peripheral reticulocyte count is low, as the marrow reticulocytes shed the reticulum while still within the marrow cavity. In general, a low peripheral reticulocyte count carries a greater degree of inaccuracy than a high count. A low count may therefore be misleading, suggesting an unfounded diagnosis of erythroid hypoplasia or aplasia.

The reticulocyte percentage can also be corrected for changes in the red blood cell volume on the basis of the hematocrit by multiplying the % reticulocyte vol. by % normal of the abnormal hematocrit.

Example:
Reticulocyte value, 25%
Hematocrit, 20 vol.%
Normal hematocrit value, 45 vol.%
Calculation:
$25 \times 20/45 = 11.1\%$ corrected reticulocyte %

The fixed marrow reticulocytes equal the circulating reticulocytes in number. In severe hemolytic processes these young marrow reticulocytes may be released into the peripheral blood and thus produce a rise in the peripheral reticulocyte count which does not actually reflect erythropoiesis. When these young reticulocytes are present, the total reticulocyte count should be divided by 2. The marrow reticulocytes can be differentiated from the more mature forms by their heavy, rich reticulum and nuclear remnants. They fall into group O-1 in Helmeyer's classification of reticulocytes which divides the cells into 4 groups according to the development of the reticulum.

The optimal normal erythropoietic marrow response to blood loss is 6-10 times normal, and in anemia the magnitude of response can be used as an indicator of bone marrow response to treatment.

Red cell utilization of radioiron (measure of effective erythropoiesis): This test includes the plasma iron turnover and the percentage of radioactivity appearing in the circulating red blood cells 2 weeks after injection (normally 75%). This method is particularly useful when the erythropoiesis is depressed and, as already stated, the reticulocyte count is inadequate. On the other hand, the radioiron utilization is inaccurate in hemolytic disease accompanied by marrow erythroid hyperplasia, a condition which can readily be appreciated by the reticulocyte count.

The **normal** is 0.45 mg./100 ml. whole blood/day.

Plasma iron turnover (PIT) (measure of total erythropoiesis): This involves the determination of plasma iron and the rate of disappearance of Fe^{59} citrate from the plasma. This method is not sensitive when erythropoiesis is decreased. It also assumes that the blood volume is normal. The **normal** plasma iron turnover is 0.6 mg./100 ml. blood/day.

Erythroid-myeloid ratio (measure of total erythropoiesis): In normal bone marrow 1 out of every 3-4 nucleated cells is a young erythroid cell. In anemia the ratio of erythroid to myeloid cells in the marrow may increase to 1:1 or even become reversed. This method is inaccurate because it depends on normal leukopoiesis and the absence of any significant dilution of the bone marrow by peripheral blood.

Destruction of red blood cells

Fecal urobilinogen: Since the degraded pigment of hemoglobin is not reutilized, the fecal urobilinogen reflects the amount of hemoglobin broken down per day. This test does not differentiate between the cells destroyed in the bone marrow or in the peripheral blood. External bleeding does not affect the measurement.

Conflicting values obtained by red cell survival studies and fecal urobilinogen may be indicative of excessive ineffective erythropoiesis.

Red cell survival: This test measures accurately the rate of loss of red cells from the circulation, but it does not indicate the pathway of loss, hemorrhage, or hemolysis. One of the best methods is the in vitro tagging of the red cells with Cr^{51} and the reinjection of the tagged cells into the patient. Young cells accept somewhat more radioactive chromium than do older cells. In practice the loss of radioactivity by elution must be taken into consideration.

Anemia-erythrokinetic classification

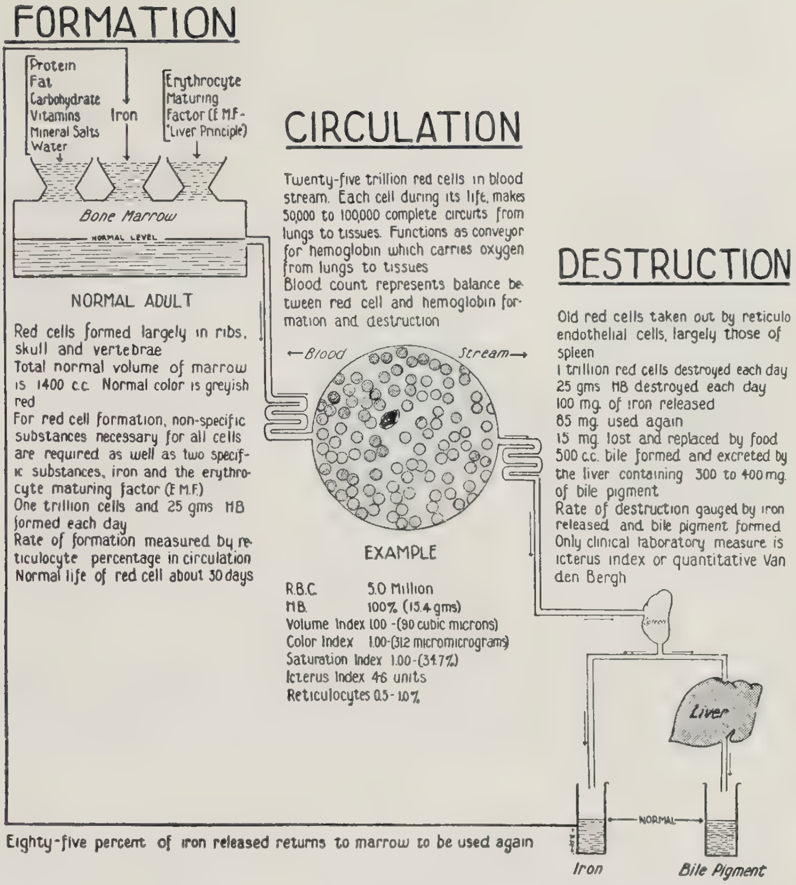
There are two main groups of anemias on this basis: anemias which lack erythroid hyperplasia of the bone marrow (nonproliferative marrow disorders) and anemias which are accompanied by myeloid erythroid proliferation (proliferative marrow disorders).

- Nonproliferative marrow disorders
  - Inadequate marrow stimulation
    - Erythropoietin deficiency
    - Renal disease
    - Endocrinopathies
    - Infection
  - Defective marrow response
    - Marrow damage from drugs, antibodies, and ionizing radiation
    - Marrow exhaustion (form of myeloproliferative disorder)
    - Anatomic replacement of marrow by tumor, fibrous tissue, etc.
- Proliferative marrow disorders
  - Dyspoietic (ineffective) proliferation
    - Abnormal nucleic acid synthesis and metabolism
      - Vitamin B_{12} deficiency
      - Folic acid deficiency
    - Abnormal hemoglobin synthesis
      - Thalassemia
      - Iron deficiency
      - Pyridoxine deficiency
      - Lead intoxication
  - Effective proliferation
    - Loss of blood due to hemorrhage
    - Loss of blood due to hemolysis

Finch, in his discussion of the erythrokinetics in the diagnosis of anemias, states that this approach stresses the site of the defect and points out whether it is premarrow, marrow, or postmarrow.[403]

Figs. 406-419 graphically present the state of the bone marrow, peripheral blood, and related organs with their relative status in normal and various abnormal conditions related to the red blood cells. Study of these illustrations is highly remunerative when related to the discussion in the text.

Text continued on p. 1244.


FORMATION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte Maturing Factor (E M.F - "Liver Principle)
Bone Marrow
NORMAL LEVEL
CIRCULATION
←Blood Stream→
DESTRUCTION
Spleen
Liver
NORMAL
Iron
Bile Pigment

NORMAL ADULT

Red cells formed largely in ribs, skull and vertebrae
Total normal volume of marrow is 1400 c.c. Normal color is greyish red
For red cell formation, non-specific substances necessary for all cells are required as well as two specific substances, iron and the erythrocyte maturing factor (E M.F.)
One trillion cells and 25 gms HB formed each day
Rate of formation measured by reticulocyte percentage in circulation
Normal life of red cell about 30 days

Twenty-five trillion red cells in blood stream. Each cell during its life, makes 50,000 to 100,000 complete circuits from lungs to tissues. Functions as conveyor for hemoglobin which carries oxygen from lungs to tissues
Blood count represents balance between red cell and hemoglobin formation and destruction

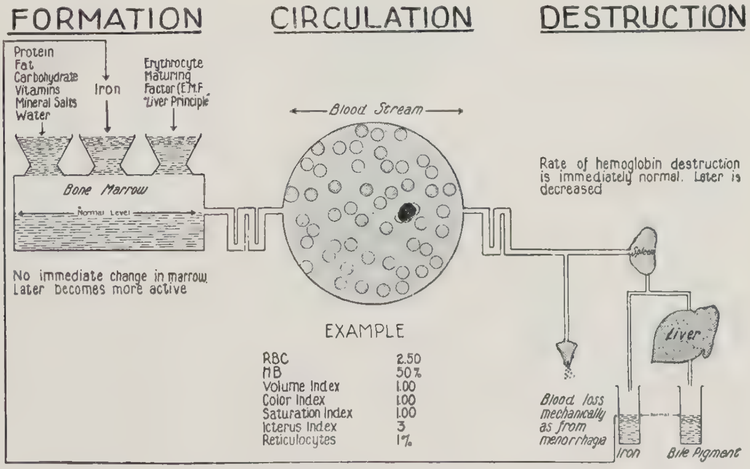
EXAMPLE

R.B.C.	5.0 Million
HB.	100% (15.4 gms)
Volume Index	1.00 -(90 cubic microns)
Color Index	1.00-(31.2 micromicrograms)
Saturation Index	1.00-(34.7%)
Icterus Index	4-6 units
Reticulocytes	0.5-1.0%

Old red cells taken out by reticulo endothelial cells, largely those of spleen
1 trillion red cells destroyed each day
25 gms HB destroyed each day
100 mg. of iron released
85 mg. used again
15 mg. lost and replaced by food
500 c.c. bile formed and excreted by the liver containing 300 to 400 mg. of bile pigment
Rate of destruction gauged by iron released and bile pigment formed
Only clinical laboratory measure is icterus index or quantitative Van den Bergh

Eighty-five percent of iron released returns to marrow to be used again

Fig. 406. Normal red cell physiology. The cells are being formed and destroyed at a constant rate. The cells vary little in volume and hemoglobin content. Rate of delivery of new cells is gauged by the reticulocyte count and the rate of destruction by the icterus index. Blood count and hemoglobin content show only the balance between formation and destruction of red cells. *Editor's note:* Normal life of the red blood cells is 120 days instead of 30 days. (From Haden, R. L.: J. Lab. & Clin. Med. **22**:439, 1937.)


FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte Maturing Factor (E.M.F "Liver Principle
Bone Marrow
Normal Level
←Blood Stream→
Spleen
Liver
Blood loss mechanically as from menorrhagia
normal
Iron
Bile Pigment

No immediate change in marrow. Later becomes more active

Rate of hemoglobin destruction is immediately normal. Later is decreased

EXAMPLE

RBC	2.50
HB	50%
Volume Index	1.00
Color Index	1.00
Saturation Index	1.00
Icterus Index	3
Reticulocytes	1%

Fig. 407. Red cells after an acute hemorrhage. The cells are reduced in number but normal in size (volume index 1) and hemoglobin content (color index 1). (From Haden, R. L.: J. Lab. & Clin. Med. **22**:439, 1937.)

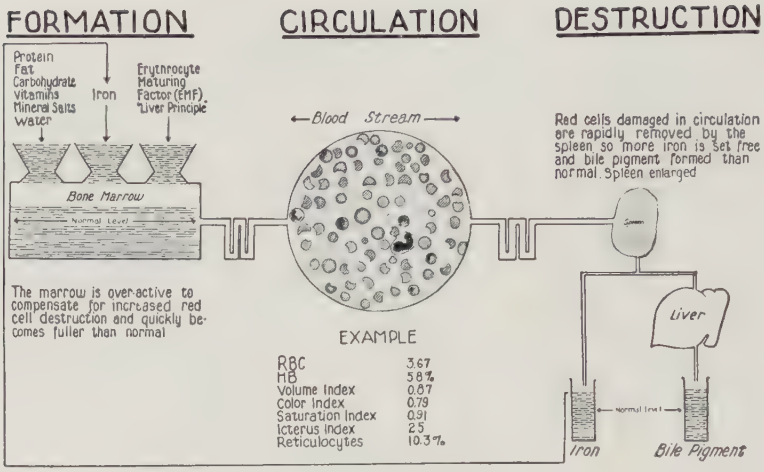

FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte Maturing Factor (EMF) "Liver Principle"
Bone Marrow
Normal Level
The marrow is over-active to compensate for increased red cell destruction and quickly becomes fuller than normal
Blood Stream
EXAMPLE
RBC 3.67
HB 58%
Volume Index 0.87
Color Index 0.79
Saturation Index 0.91
Icterus Index 25
Reticulocytes 10.3%
Red cells damaged in circulation are rapidly removed by the spleen so more iron is set free and bile pigment formed than normal. Spleen enlarged
Liver
Normal level
Iron
Bile Pigment

Fig. 408. Red cells in excessive destruction by a toxic substance (phenylhydrazine). Red cells are damaged and then rapidly removed from circulation by the spleen, so that splenomegaly results. High icterus index (25 units) is due to accelerated destruction of hemoglobin. High reticulocyte count (10.3%) is an attempt on the part of the bone marrow to compensate for abnormal destruction of cells. (From Haden, R. L.: J. Lab. & Clin. Med. **22**:439, 1937.)

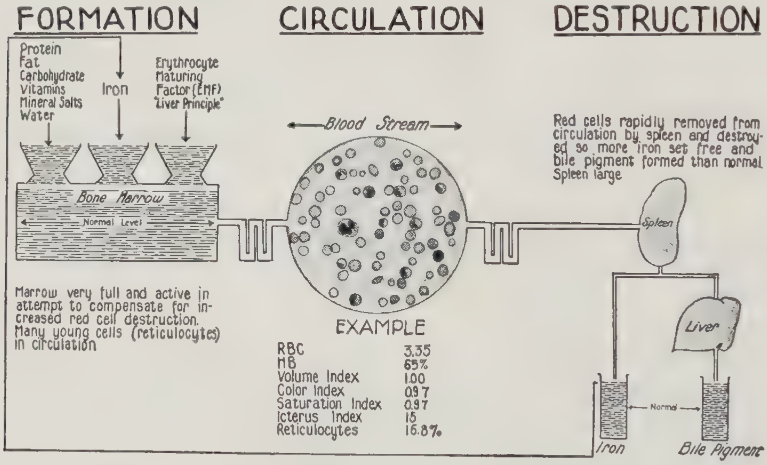

FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte Maturing Factor (EMF) "Liver Principle"
Bone Marrow
Normal Level
Marrow very full and active in attempt to compensate for increased red cell destruction. Many young cells (reticulocytes) in circulation
Blood Stream
EXAMPLE
RBC 3.35
HB 65%
Volume Index 1.00
Color Index 0.97
Saturation Index 0.97
Icterus Index 15
Reticulocytes 16.8%
Red cells rapidly removed from circulation by spleen and destroyed so more iron set free and bile pigment formed than normal. Spleen large
Spleen
Liver
Normal
Iron
Bile Pigment

Fig. 409. Red cells with excessive removal from the blood stream by the spleen because of spherocytic shape. In congenital hemolytic icterus the cells are abnormal in shape (spherocytes) and are rapidly removed from the circulation. Icterus index is high (15 units) due to the excessive destruction, and the reticulocyte per cent is also high (15.8%), indicating an excessive release of red cells from the marrow. In this condition the cells remain in the circulation only a few days as compared with a normal time of 120 days. (From Haden, R. L.: J. Lab. & Clin. Med. **22**:439, 1937.)

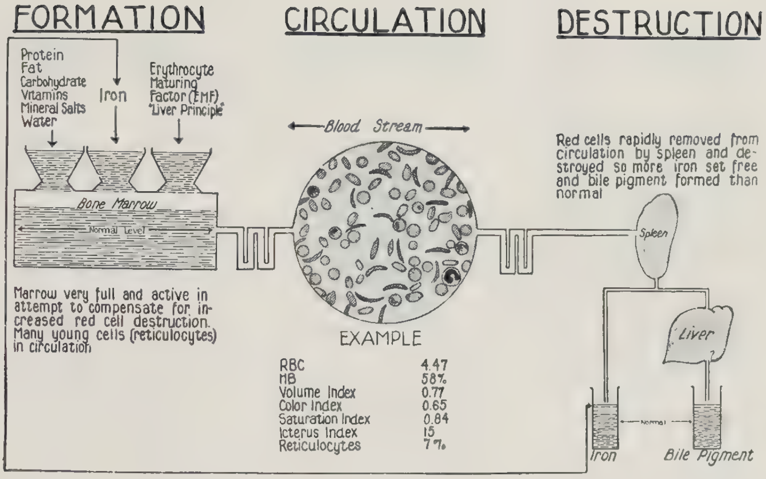

FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte
Maturing
Factor (EMF)
"Liver Principle"
Bone Marrow
Normal Level
Marrow very full and active in attempt to compensate for increased red cell destruction. Many young cells (reticulocytes) in circulation
Blood Stream
EXAMPLE
RBC 4.47
HB 58%
Volume Index 0.77
Color Index 0.65
Saturation Index 0.84
Icterus Index 15
Reticulocytes 7%
Red cells rapidly removed from circulation by spleen and destroyed so more iron set free and bile pigment formed than normal
Spleen
Liver
Normal
Iron
Bile Pigment

Fig. 410. Red cells in excessive removal from the blood stream by the spleen because of sickle shape. This is also a hemolytic anemia as shown by increased icterus index and reticulocytes. (From Haden, R. L.: J. Lab. & Clin. Med. **22**:439, 1937.)

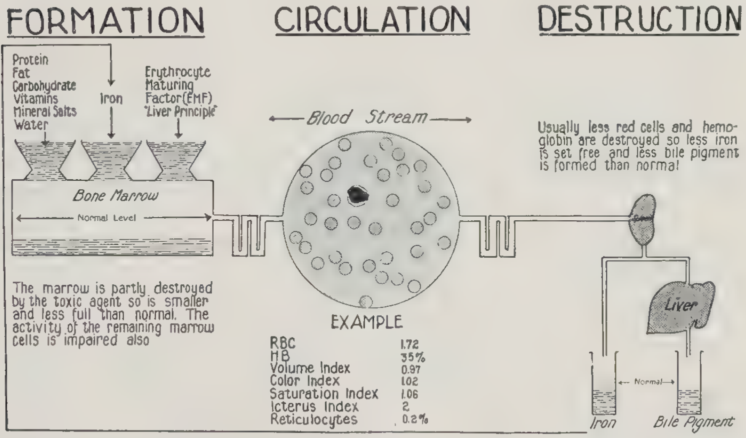

FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte
Maturing
Factor (EMF)
"Liver Principle"
Bone Marrow
Normal Level
The marrow is partly destroyed by the toxic agent so is smaller and less full than normal. The activity of the remaining marrow cells is impaired also
Blood Stream
EXAMPLE
RBC 1.72
HB 35%
Volume Index 0.97
Color Index 1.02
Saturation Index 1.06
Icterus Index 2
Reticulocytes 0.2%
Usually less red cells and hemoglobin are destroyed so less iron is set free and less bile pigment is formed than normal
Liver
Normal
Iron
Bile Pigment

Fig. 411. Red cells in anemia due to aplasia of marrow by myelotoxic agent such as arsphenamine. Here the marrow is decreased in volume and few erythrocytes, leukocytes, or thrombocytes can be delivered. Such cells as are found are normal. (From Haden, R. L.: J. Lab. & Clin. Med. **22**:439, 1937.)

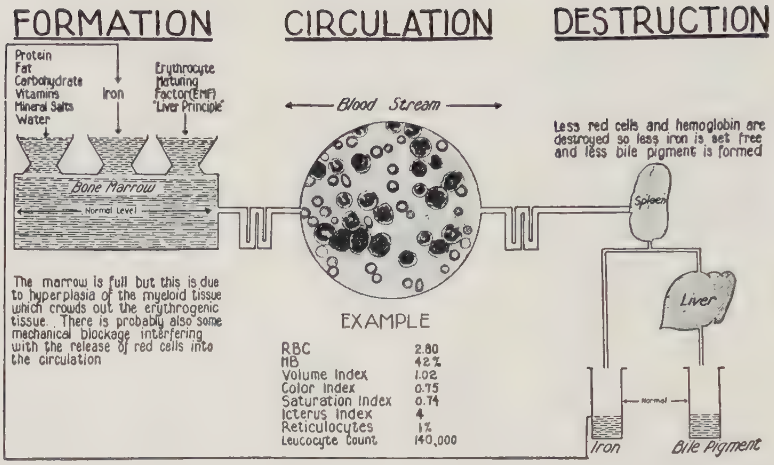

FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte
Maturing
Factor(EMF)
"Liver Principle"
Bone Marrow
Normal Level
Blood Stream
Less red cells and hemoglobin are destroyed so less iron is set free and less bile pigment is formed
Spleen
Liver
The marrow is full but this is due to hyperplasia of the myeloid tissue which crowds out the erythrogenic tissue. There is probably also some mechanical blockage interfering with the release of red cells into the circulation
EXAMPLE
RBC 2.80
HB 42%
Volume Index 1.02
Color Index 0.75
Saturation Index 0.74
Icterus Index 4
Reticulocytes 1%
Leucocyte Count 140,000
Normal
Iron
Bile Pigment

Fig. 412. Red cell in a quantitative depression of bone marrow as by leukemia. Here the erythropoietic tissue is crowded out by the leukopoietic tissue so that less cells than normal can be formed. Such cells as are formed are normal in volume and hemoglobin content. (From Haden, R. L.: J. Lab. & Clin. Med. **22**:439, 1937.)

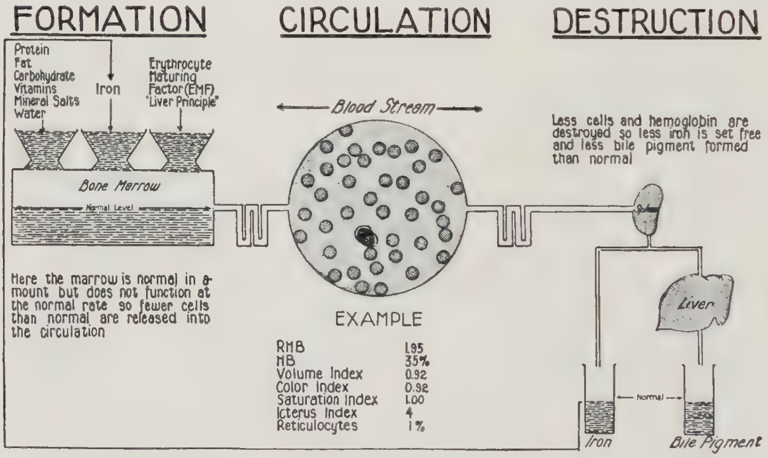

FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte
Maturing
Factor (EMF)
"Liver Principle"
Bone Marrow
Normal Level
Blood Stream
Less cells and hemoglobin are destroyed so less iron is set free and less bile pigment formed than normal
Liver
Here the marrow is normal in amount but does not function at the normal rate so fewer cells than normal are released into the circulation
EXAMPLE
RHB 1.95
HB 35%
Volume Index 0.92
Color Index 0.92
Saturation Index 1.00
Icterus Index 4
Reticulocytes 1%
Normal
Iron
Bile Pigment

Fig. 413. Red cell in a quantitative depression of bone marrow as by nephritis. Here the amount of functioning tissue is not decreased, but the rate of activity is much less than normal. Again the cells formed are normal in volume and hemoglobin content. (From Haden, R. L.: J. Lab. & Clin. Med. **22**:439, 1937.)

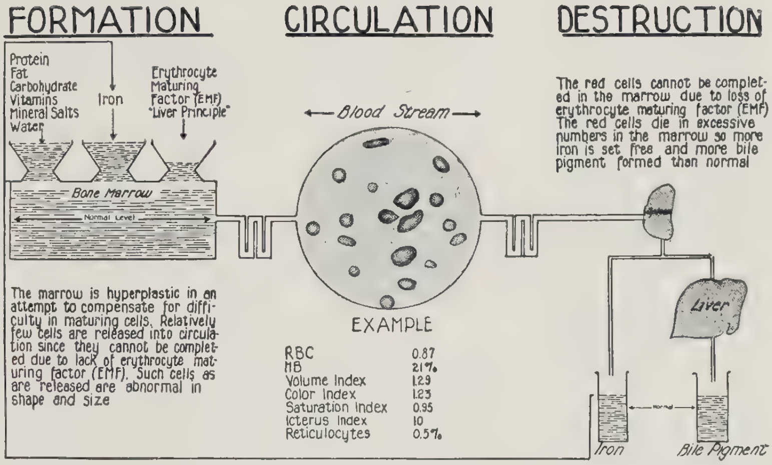

FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte Maturing Factor (EMF) "Liver Principle"
Bone Marrow
Normal Level
The marrow is hyperplastic in an attempt to compensate for difficulty in maturing cells. Relatively few cells are released into circulation since they cannot be completed due to lack of erythrocyte maturing factor (EMF). Such cells as are released are abnormal in shape and size
Blood Stream
EXAMPLE
RBC 0.87
HB 21%
Volume Index 1.29
Color Index 1.23
Saturation Index 0.95
Icterus Index 10
Reticulocytes 0.5%
The red cells cannot be completed in the marrow due to loss of erythrocyte maturing factor (EMF) The red cells die in excessive numbers in the marrow so more iron is set free and more bile pigment formed than normal
Liver
Normal
Iron
Bile Pigment

Fig. 414. Red cells in an anemia due to an idiopathic deficiency in erythrocyte-maturing factor (pernicious anemia). Here the marrow is hyperplastic because the cells that cannot be normally completed accumulate. Such cells as are released are not normal as shown by the macrocytosis (volume index 1.29) which indicates a deficiency of specific-maturing factor (EMF). Many cells die in the marrow since they are not normally released, so that the bile pigment content is increased (icterus index, 10 units). (From Haden, R. L.: J. Lab. & Clin. Med. 22:439, 1937.)

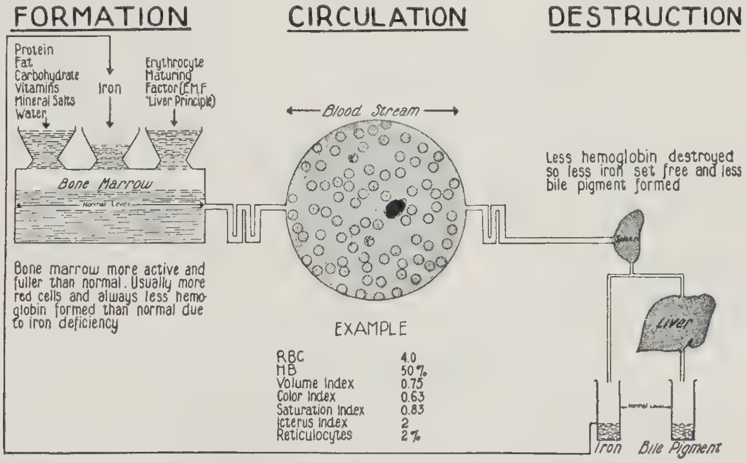
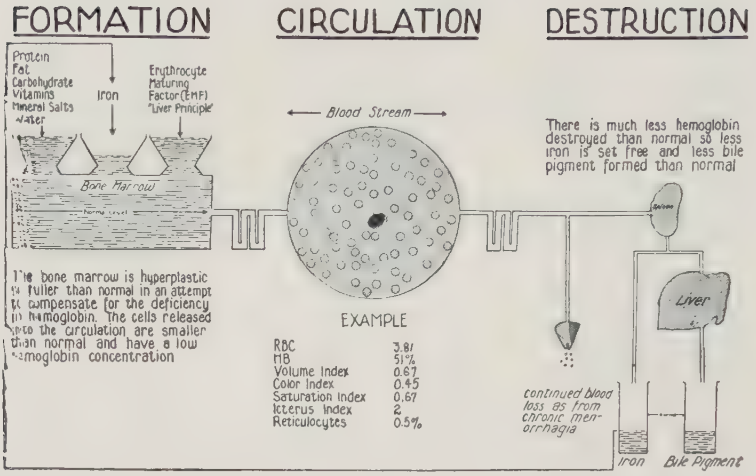

FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte Maturing Factor (E.M.F "Liver Principle)
Bone Marrow
Normal Level
Bone marrow more active and fuller than normal. Usually more red cells and always less hemoglobin formed than normal due to iron deficiency
Blood Stream
EXAMPLE
RBC 4.0
HB 50%
Volume Index 0.75
Color Index 0.63
Saturation Index 0.83
Icterus Index 2
Reticulocytes 2%
Less hemoglobin destroyed so less iron set free and less bile pigment formed
Liver
Normal Level
Iron
Bile Pigment

Fig. 415. Red cells with a deficient intake of iron. As a result of the chronic iron deficiency, the hemoglobin is reduced (color index 0.63) and the cells become smaller (volume index 0.75). Icterus index is low because less hemoglobin is destroyed. Reduction in number of cells is much less than in hemoglobin. (From Haden, R. L.: J. Lab. & Clin. Med. 22:439, 1937.)


FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte Maturing Factor (EMF) "Liver Principle"
Bone Marrow
Normal Level
Blood Stream
Spleen
Liver
Iron
Bile Pigment

The bone marrow is hyperplastic or fuller than normal in an attempt to compensate for the deficiency in hemoglobin. The cells released into the circulation are smaller than normal and have a low hemoglobin concentration

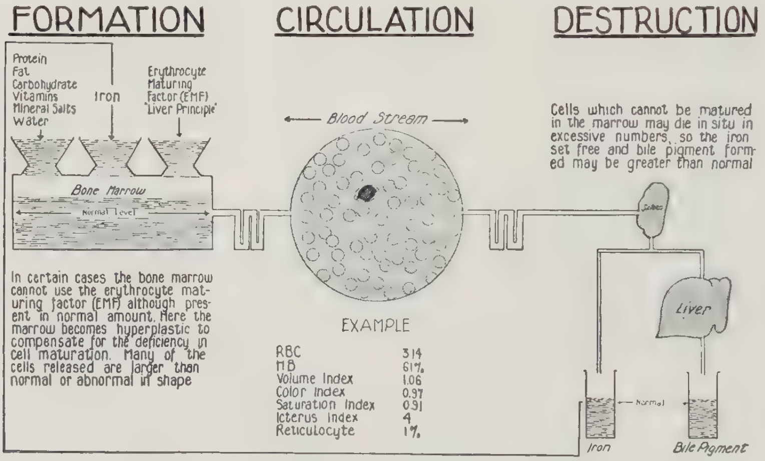
There is much less hemoglobin destroyed than normal so less iron is set free and less bile pigment formed than normal

continued blood loss as from chronic menorrhagia

EXAMPLE

RBC	3.81
HB	51%
Volume Index	0.67
Color Index	0.45
Saturation Index	0.67
Icterus Index	2
Reticulocytes	0.5%

Fig. 416. Red cells with chronic bleeding. Here there is a chronic iron deficiency and the picture is exactly like that with a deficient intake of iron (Fig. 415). (From Haden, R. L.: J. Lab. & Clin. Med. **22**:439, 1937.)


FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte Maturing Factor (EMF) "Liver Principle"
Bone Marrow
Normal Level
Blood Stream
Spleen
Liver
Normal
Iron
Bile Pigment

In certain cases the bone marrow cannot use the erythrocyte maturing factor (EMF) although present in normal amount. Here the marrow becomes hyperplastic to compensate for the deficiency in cell maturation. Many of the cells released are larger than normal or abnormal in shape

Cells which cannot be matured in the marrow may die in situ in excessive numbers, so the iron set free and bile pigment formed may be greater than normal

EXAMPLE

RBC	3.14
HB	61%
Volume Index	1.06
Color Index	0.97
Saturation Index	0.91
Icterus Index	4
Reticulocyte	1%

Fig. 417. Red cells in a qualitative depression of marrow in which erythrocyte-maturing factor is not normally used. Here the marrow receives the specific factor but cannot use it. Picture is similar to that with an idiopathic defect in formation of the necessary factor (Fig. 414). (From Haden, R. L.: J. Lab. & Clin. Med. **22**:439, 1937.)

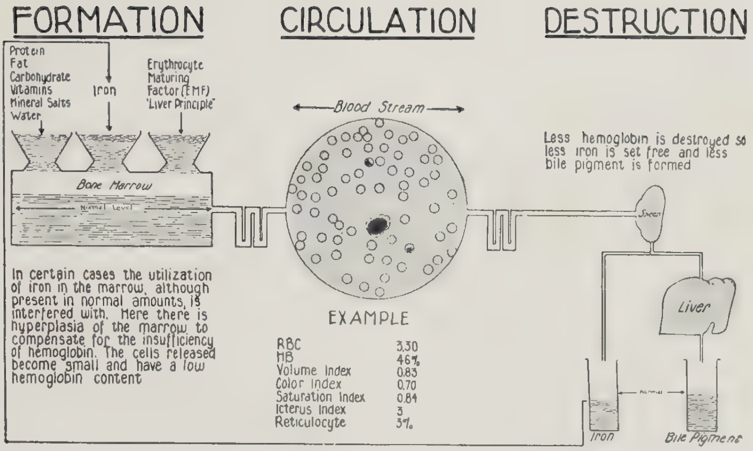

FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrate
Vitamins
Mineral Salts
Water
Iron
Erythrocyte Maturing Factor (EMF) "Liver Principle"
Blood Stream
Bone Marrow
Normal Level
In certain cases the utilization of iron in the marrow, although present in normal amounts, is interfered with. Here there is hyperplasia of the marrow to compensate for the insufficiency of hemoglobin. The cells released become small and have a low hemoglobin content
EXAMPLE
RBC 3.30
HB 46%
Volume Index 0.83
Color Index 0.70
Saturation Index 0.84
Icterus Index 3
Reticulocyte 3%
Less hemoglobin is destroyed so less iron is set free and less bile pigment is formed
Spleen
Liver
Normal
Iron
Bile Pigment

Fig. 418. Red cells in a qualitative depression of marrow in which iron cannot be used. Here the marrow receives iron but cannot incorporate it normally so that the picture is similar to that due to an insufficient supply of iron (Figs. 415 and 416). (From Haden, R. L.: J. Lab. & Clin. Med. 22:439, 1937.)

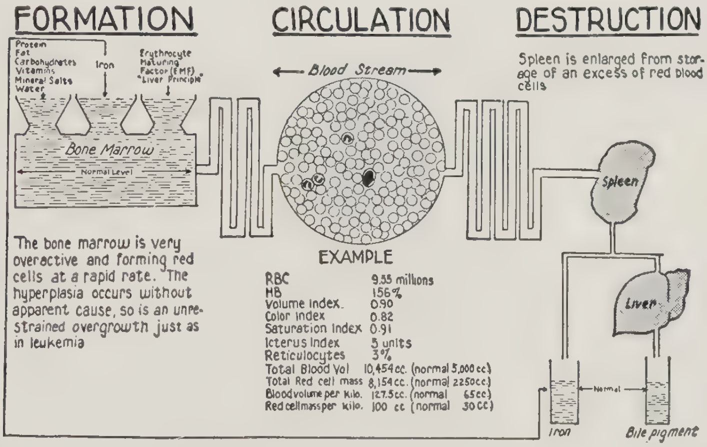

FORMATION
CIRCULATION
DESTRUCTION
Protein
Fat
Carbohydrates
Vitamins
Mineral Salts
Water
Iron
Erythrocyte Maturing Factor (EMF) "Liver Principle"
Blood Stream
Spleen is enlarged from storage of an excess of red blood cells
Bone Marrow
Normal Level
The bone marrow is very overactive and forming red cells at a rapid rate. The hyperplasia occurs without apparent cause, so is an unrestrained overgrowth just as in leukemia
EXAMPLE
RBC 9.55 millions
HB 156%
Volume Index. 0.90
Color Index 0.82
Saturation Index 0.91
Icterus Index 5 units
Reticulocytes 3%
Total Blood Vol 10,454 cc. (normal 5,000 cc.)
Total Red cell mass 8,154 cc. (normal 2250 cc.)
Bloodvolume per kilo. 127.5 cc. (normal 65 cc.)
Red cellmassper kilo. 100 cc (normal 30 CC)
Spleen
Liver
Normal
Iron
Bile pigment

Fig. 419. Red cells in polycythemia vera. In this disease there is an enormous overproduction of erythrocytes and the marrow is filled with erythrogenic tissue. Rate of destruction does not keep pace with red cell formation and the blood volume increases. Increase in red cells per unit volume and in blood volume gives a great increase in red cell mass. (From Haden: R. L.: Principles of hematology, Philadelphia, Lea & Febiger.)

MICROCYTIC ANEMIAS

Iron-deficiency anemia (sideropenia)

The most common type of anemia is iron-deficiency anemia. The well-established anemia is hypochromic microcytic and shows some anisocytosis and poikilocytosis.

This anemia is closely linked to iron metabolism (ferrokinetics), and therefore this aspect is discussed briefly first.

Iron metabolism

In the normal adult (70 kg.) there are about 4-5 gm. iron which have the following distribution: hemoglobin 65-75%, storage depots (liver, spleen, and bone marrow) 15-25%, myoglobin 5%, and enzymes (cytochromes, catalase, etc.), 5%. Alimentary iron is absorbed mainly in the duodenum, some in the jejunum, and hardly any in the stomach, ileum, and colon. In the stomach alimentary iron is broken down to ionized ferric iron (Fe^{+++}) which is then reduced to ferrous iron (Fe^{++}) by ascorbic acid, cysteine, and the sulfhydryl groups of food proteins. Although Moore and others have shown that hydrochloric acid is not essential for ionizing alimentary iron, many European investigators believe that achlorhydria interferes with effective iron absorption. About 5-10% of the alimentary iron is absorbed. If the diet contains 12-15 mg. iron/day, 0.6-1.5 mg. will be absorbed. The mechanism and the control of iron absorption are not clear. There are many good arguments against Granick's mucosal block theory which postulates that the intracellular concentration of ferritin within the duodenal mucosa regulates the iron absorption. In diseases characterized by high serum iron the iron absorption continues at the normal rate. Saltman showed that the iron transfer may be in part a chelating process depending (1) on the total number of available binding sites of ferritin or transferrin, (2) on the stability of each of these sites, and (3) on the competition of other ions for these sites. The iron absorption depends on several factors. Iron in various foods is not equally well absorbed. Iron deficiency and marrow erythropoietic hyperplasia increase the amount of absorbed iron several times (20%), whereas marrow erythropoietic hypoplasia and large iron deposits in the storage organs tend to retard iron absorption. It is further delayed by increased intestinal motility and by diets con-

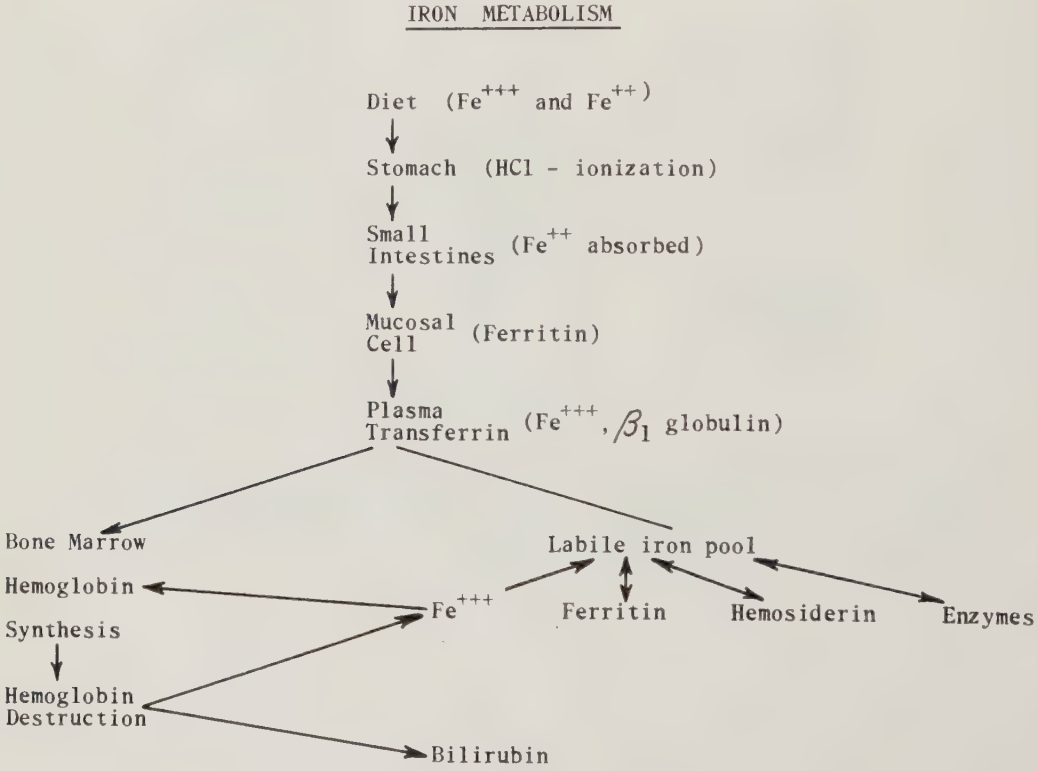

IRON METABOLISM
Diet (Fe+++ and Fe++)
Stomach (HCl - ionization)
Small Intestines (Fe++ absorbed)
Mucosal Cell (Ferritin)
Plasma Transferrin (Fe+++, β1 globulin)
Bone Marrow
Hemoglobin
Synthesis
Hemoglobin Destruction
Fe+++
Bilirubin
Labile iron pool
Ferritin
Hemosiderin
Enzymes

Fig. 420. Outline of iron metabolism. (From Bauer, J. D., Toro, G., and Ackermann, P. G.: Bray's clinical laboratory methods, St. Louis, 1962, The C. V. Mosby Co.)

taining large amounts of certain organic acids such as lactic acid.

In the process of absorption the ferrous iron is oxidized to the ferric state and is bound to an α-2 globulin, apoferritin, the iron complex of which is called ferritin. Apoferritin is rapidly synthesized and is equally rapidly broken down in the mucosal epithelial cells. The ferric form is again reduced to the ferrous form and diffuses into the plasma where it circulates as Fe^{+++} bound to a β globulin, transferrin or siderophilin. This protein is normally only one third saturated with iron. Iron is thus transported to the storage depots—bone marrow, liver, spleen—but it is mainly utilized for hemoglobin synthesis. Small amounts are incorporated into enzymes and myoglobin. In hemoglobin the iron is present in a mineralized form.

The body carefully conserves iron by reutilizing iron which is liberated in the process of cellular catabolism (especially hemoglobin iron). Iron is stored as ferritin which contains iron as $Fe(OH)_3$ and does not stain with Prussian blue dye. Excess iron is stored as hemosiderin which is a protein, containing nucleotides and iron as iron hydroxide gel. Hemosiderin stains blue with Prussian blue. Hemosiderin may play some role in the cellular defense mechanism. This process is reversible so that iron can be transferred from siderophilin to ferritin and hemosiderin and vice versa. Iron deficiency does not therefore occur until all 3 proteins are depleted of iron.

Most of the alimentary iron is excreted in feces. A small amount of iron is lost in urine, sweat, and cutaneous and intestinal desquamating cells. Parenterally administered iron is not excreted. Blood loss from any source, whether physiologic such as menstruation or pathologic such as hemorrhage, adds to the iron loss. The average daily loss for adult men and postmenopausal women is 0.5-1 mg. iron. In menstruating women the loss increases to 1-1.5 mg. These figures roughly represent the daily minimal iron requirements of these 3 groups. During pregnancy and delivery the iron loss of a mother is in the neighborhood of 100-500 mg. In childhood during the growth periods, 0.6 mg. of iron must be absorbed daily in excess of the normal requirements.

The **erythrokinetic data** in a normal man (70 kg.) are as follows: blood volume, 5000 ml.; red cell mass, 2000 ml.; daily turnover of red cells, 16.7-20 ml. These figures correspond to a hemoglobin mass of 666 gm. and a daily hemoglobin turnover of 5.6-6.7 gm., which in turn corresponds to a circulating iron mass of 2254 mg. and a daily iron turnover of 18.5-22.5 mg. The depot iron amounts to about 1000-1500 mg.

The investigation of human ferrokinetics is made possible by the administration of radioiron (Fe^{59}) which allows measurement of (1) radioiron utilization for hemoglobin formation, (2) of plasma volume (in conjunction with P^{32} of red cell volume), and (3) determination of circulating iron and iron storage and excretion.

Laboratory findings

Hematologic findings

Peripheral blood:

RBC: decreased (3-4 million/c.mm.).

Hemoglobin: decreased (6-10 gm./100 ml.).

Constants

MCV less than 80 μ^3.

MCH less than 27 $\mu\mu$g.

MCHC less than 32%.

Blood films: Hypochromasia, microcytosis, planocytosis, anulocytosis, occasional target cells, and anisocytosis.

Reticulocytes: Usually decreased; most are young.

Osmotic fragility: Normal to decreased (presence of target cells).

White blood cell count: Normal; often slight leukopenia with lymphocytosis; granulocytes may be hypersegmented and small.

Platelets: Normal; sometimes slightly diminished.

Serum iron: Low, less than 60 μg/100 ml. blood; total serum binding capacity high, greater than 350 μg/100 ml.; rapid response to parenteral iron administration.

A single serum iron determination is to be interpreted with caution because of the great normal variations of serum iron. Radioiron shows rapid absorption, rapid removal from plasma associated with rapid uptake by the bone marrow, and slight shortening of the life span of red cells. There is minimal radioiron excretion.

The diagnosis of depleted iron stores can be made by the iron absorption test. After an iron test dose of about 170 mg. Fe, a rise of serum iron over 280 mg.% after 2 hr. is indicative of iron deficiency.

Bone marrow: Increase in basophilic normoblasts which may show nuclear cytoplasmic dissociation due to nuclear condensation

Table 246. *The anemias**

Terms to be used	*Terms to be avoided*
Normocytic anemia due to:	
Unknown cause	Refractory anemia, secondary anemia
Hypoplastic normocytic anemia (pancytopenia), due to	
Unknown cause	Idiopathic aplastic anemia, panmyelophthisis, aregeneratory anemia, aleukia haemorrhagica, toxic paralytic anemia, cryptogenic anemia, refractory anemia, pancytopenia, Felty's syndrome
Congenital	Fanconi's syndrome, constitutional infantile anemia
Poison (specify, as benzol)	
Infection	Simple chronic anemia, secondary anemia
Radiation from:	
Roentgen rays	
Radioactive substances	
Osteopetrosis	Marble bone disease, Albers-Schönberg disease
Myelofibrosis	
Congestive splenomegaly due to:	
Unknown cause	Banti's disease, splenic anemia
Thrombosis of splenic or portal vein	
Liver disease	
Normocytic anemia with erythrocytic hypoplasia due to:	
Unknown cause	Pseudoaplastic anemia, refractory anemia, progressive hypocythemia, progressive erythrophthisis
Congenital	
Endocrine hypofunction	
Hypothyroidism	
Poison	
Infection	
Normocytic anemia with myelophthisis due to:	
Unknown cause	Leukoerythroblastosis, myelopathic anemia, metastatic anemia
Osteopetrosis	Osteopathia condensans disseminata, chronic nonleukemic myelosis, Albers-Schönberg disease, marble bone disease
Myelofibrosis	
Amyloidosis	
Metastatic neoplasm	
Leukemia	
Plasmacytic myeloma	
Normocytic anemia from internal destruction of erythrocytes due to:	
Unknown cause	Hemolytic anemia
Poison	
Lead	
Infection	
Parasite	
Malaria	
Sensitization to Rh-Hr or other erythrocytic factors	
Hemolytic disease of (fetus and) newborn	Erythroblastosis fetalis, erythroleukoblastosis, icterus gravis neonatorum, hydrops fetalis
Transfusion hemolysis	
Internal hemorrhage	
Hemoglobinuria	
Unknown cause	
Paroxysmal hemoglobinuria	Hemoglobinemia
Unknown cause	
Nocturnal	Marchiafava-Micheli syndrome
Cold	"e frigore"
Exertional	March hemoglobinuria
Allergic	
Favism	
Hemolytic normocytic anemia, acquired due to:	
Unknown cause	
Acute	Lederer's anemia, Winckel's disease, acute febrile pleochromic anemia
Cold agglutinins	
Sulfonamide sensitivity	
Leukemia, lymphocytic	
Ovarian cyst	

*From Am. J. Clin. Path. 20:562-571, 1950.

Table 246. *The anemias*—cont'd

Terms to be used	*Terms to be avoided*
Hemolytic normocytic anemia, hereditary due to:	
Hereditary spherocytosis	Familial hemolytic icterus, acholuric jaundice, congenital hemolytic icterus, hemolytic icteroanemia
Trait	
Hereditary ovalocytosis	
Trait	
Sicklemia	Sicklanemia, drepanocytic anemia, Dresbach's syndrome, Herrick's anemia
Trait	
Normocytic anemia due to:	
Acute blood loss	
Metabolic disturbance from:	
Unknown cause	
Lipid histiocytosis	
Protein deficiency	
Vitamin deficiency	
Sprue	
Hypochromic microcytic anemia due to:	Secondary anemia, chlorosis
Unknown cause	Idiopathic anemia, simple achlorhydric anemia, Witt's anemia, chronic microcytic anemia, idiopathic hypochromemia, chronic chlorosis, erythronormoblastic anemia
Chronic blood loss	
Deficient intake, absorption, or metabolism of iron	Secondary anemia, hypoferric anemia, achromic anemia, simple anemia, iron deficiency anemia
Prematurity	
Hereditary leptocytosis	Cooley's anemia, thalassemia, erythroblastic anemia, Mediterranean anemia, disease, or fever, target cell anemia, familial microcytic anemia, hereditary or familial poikilocytosis
Trait	
With erythrocytosis	
Pernicious anemia, type *macrocytic anemia* due to:	
Unknown cause	Achestic anemia, idiopathic refractory megaloblastic anemia
Infantile	Megaloblastic anemia of infancy
Pernicious anemia	Addisonian anemia, Biermer's disease, primary anemia, macrocytic anemia, hyperchromic anemia, megalocytic anemia, megaloblastic anemia
Pregnancy	Severe anemia of pregnancy, primary anemia of pregnancy, hemolytic anemia of pregnancy, macrocytic anemia of pregnancy
Sprue	
Tropical	Indische Sprue, psilosis, aphthae tropicae, tisis intestinal, Cochin-China diarrhea
Nontropical	Idiopathic steatorrhea, Gee-Herter disease
Nutritional deficiency	Goat's milk anemia, tropical macrocytic anemia
Celiac disease	
Chronic intestinal obstruction	
Diphyllobothriasis	
Gastrectomy	
Disturbance in continuity of gastrointestinal tract	
Tuberculous enteritis	
Macrocytic anemia due to:	
Unknown cause	Achrestic anemia
Hypoplasia	
Liver disease	

preceding cytoplasmic hemoglobinization. The cytoplasm appears to be mechanically weak so that the edges are irregular, and often naked nuclei appear (ineffective erythropoiesis). In the storage organs, including the bone marrow, hemosiderin is absent. The absence of bone marrow hemosiderin is an important finding in iron deficiency. Because iron returns slowly into the depleted storage depots, the absence of bone marrow hemosiderin is a diagnostic finding in latent sideropenia[404] (masked iron deficiency) in which case the patient suffers from iron-deficiency anemia, but the serum iron and the hemoglobin values are only slightly depressed.

Nonhematologic findings

Frequently gastric achylia is found which is occasionally histamine fast. There may be atrophy of the epithelial cells of the gastrointestinal tract (cytochrome deficiency?), and oral epithelial cornification has been described. Rarely bone changes can be seen on radiologic examination.

Causes of iron-deficiency anemia

Following is a list of the main causes of iron deficiency.

1. Insufficient intake, which includes poor dietary habits, inadequate diet during childhood, and reducing diets.
2. Inadequate absorption, which includes achlorhydria (?), extensive gastric resection, substances interfering with absorption such as citric acid and lactic acid, lack of ascorbic acid, accelerated passage as in chronic diarrhea, and colitis.
3. Increased demand, which occurs during pregnancy, in growing children, and from blood loss and blood regeneration.
4. Blood loss, which includes physiologic loss during menses, and pathologic as from hemorrhage and hemolysis.
5. Abnormal intrinsic iron metabolism, which occurs in pulmonary hemosiderosis and sideroachrestic anemia.

Insufficient intake

Except in extreme cases of general malnutrition or malabsorption, insufficient intake is not the cause of iron deficiency in adults.

Iron deficiency in infants and children: In infants and children iron deficiency is a common cause of anemia. Physiologically, from birth on the hemoglobin and number of red blood cells fall rapidly up to 3 mo. so that the storage depots become filled with iron from the breakdown of red blood cells. From 3 mo. on the depots become gradually depleted if the infant is on a pure milk diet, as milk alone does not supply enough iron to restore the depot iron. This physiologic(?) iron deficiency is even more marked if the child is premature or is one of twins, if the placental blood was not allowed to drain into the infant at the time of birth (cesarean section), or if the infant bled into the maternal circulation[405] or was born after placental hemorrhage had occurred. Iron-deficiency anemia is common at the end of the first or second year, after which time the iron storage depots are slowly replenished.

Chlorosis

Chlorosis is of historic interest only. The name refers to a form of perhaps hereditary iron-deficiency anemia often accompanied by achlorhydria which had its peak in 1890, 1900, and 1901 in Europe and has since disappeared. The cause of its disappearance is unknown and is perhaps related to dietary and social habits. Heilmeyer made the remark that many families' recipes are also hereditary.

Chlorosis is a very interesting disease frequently seen in young women although Castle and Minot[406] state: "At present it is very rarely observed at least in the form described in the medical literature of the preceding three centuries. Undoubtedly cases of hypochromic anemia in young women, recognized today as associated with defective diets, decreased gastric secretions, and chronic blood loss, would have been called chlorosis a generation ago."* They exhibit symptoms of weakness. These patients will complain in the morning after a good night's sleep of not being refreshed by sleep, but in the afternoon and toward the evening this weakened condition disappears. They are not necessarily pale. They complain of a tired feeling, headache, difficulty in breathing after bodily exercise, palpitation of the heart, pain in the side, and disturbances of menstruation. Dyspeptic symptoms are frequent.

They are often pale but not necessarily so. The lips and visible mucosae are pale, but the cheeks are not. The skin color may be pale yellow or pale green.

Clinical symptoms are as just described, plus dark spots floating before the eyes, disturbances in accommodation, and quick fatigue after reading. The retina is pale and is characterized by transparency of the blood vessels. There may be spasms with cold hands and feet. Thromboses have been described in this disease. The pulse is full, soft, and dicrotic. Blood pressure is normal. The spleen is not necessarily enlarged, according to Schilling, although von Noorden states that it is always enlarged.

Blood loss

Hemorrhagic and posthemorrhagic anemia: Certain changes in the blood picture are of importance in estimating the extent of the hemorrhage and the amount of attempted regeneration on the part of the erythropoietic and leukopoietic systems.

A rapidly rising erythrocyte count, i.e., a constant rise if counts are taken hourly or periodically during the day, denotes a possible hemoconcentration leading to shock.

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*From Castle, W. B., and Minot, G. R.: Pathological physiology and clinical description of the anemias, New York, 1936, Oxford University Press.

Leukocytosis with neutrophilia and no shift is seen following hemorrhages. In severe hemorrhage there is a slight left shift. In exhausting hemorrhages there is a regenerative shift.

Marked polychromasia in the blood film (rise in reticulocytes) indicates regeneration of the erythrocytes and is a good sign following a hemorrhage. Basophilic punctation means degenerative effects, possibly toxic, on the erythrocytes. An occasional normoblast can be found in the blood in severe hemorrhages as a sign of rapid regeneration of the erythrocytes, but if many normoblasts appear, there is overstimulation of the erythropoietic system, and this is regarded unfavorably.

Blood platelets rise rapidly following a hemorrhage.

Roughly 50 mg. Fe are lost per 100 ml. blood. The hemorrhage may be intermittent and may be so small in amount as to be difficult to detect and may come from hidden lesions which may be difficult to diagnose such as diverticula, hiatus hernia, etc. Excessive menstrual loss, frequent pregnancies, and frequent blood donations to blood banks all may account for iron loss.

In general the blood count immediately following hemorrhage will not reflect the blood loss. As the organism attempts to compensate for the blood loss by mobilizing interstitial fluid, which is poor in protein, the blood count and the serum protein will fall. This fall may continue for several days even though the hemorrhage has ceased. The normal iron depots contain about 1000 mg. iron, which is enough to compensate for the loss of 1 or 2 L. blood by mobilizing 20-40 mg. a day. If the blood loss is sudden and of any magnitude, young marrow reticulocytes together with nucleated cells are released into the circulation. Bone marrow erythroid hyperplasia will follow within a matter of hours and days. If the blood loss continues, the erythropoiesis will extend into fatty marrow, replace it, and expand the marrow-containing spaces. Potentially erythropoietic organs such as the spleen, liver, lymph nodes, and retroperitoneal fat (extramedullary metaplastic erythropoiesis or hematopoiesis) are called into play.

Hookworm anemia

The anemia is a typical iron-deficiency anemia, related to the severity of the infection, the nutritional state of the patient, and the coexisting iron (blood) losses, such as menses. It is reported that each worm consumes 0.67 gm. hemoglobin daily. Additional factors are the nutritional hypoproteinemia and the malabsorption related to the hyperperistalsis of the diarrhea.

Hookworm anemia has been known since the construction of the Gotthard tunnel in Switzerland in the years 1876-1880. Perroncito[407] noted that many Italian workers developed a severe and at times fatal anemia, with hookworm infestation. Later, hookworm anemia was called miner's anemia because of its occurrence in mines. Boycott[408] described cases of hookworm anemia in England (1901). Later hookworm anemia was observed in other European countries, in America, and in tropical countries where suitable conditions for hookworm infestation exist.

The etiology of this disease has been well discussed in many papers. Stransky and Quintos[409] gave an accurate description of it from their experience in the Philippines, where the Ancylostoma is very common.

They observed 3 types or stages of blood changes in hookworm disease. In the first type the bone marrow is able to maintain the equilibrium between blood loss and production, and there is no manifest anemia. They call this the stage of compensated anemia. In the second type the production is unable to counterbalance the blood loss, and a hypochromic anemia results. In the third type the bone marrow is exhausted, and aplastic anemia develops. In the first type they found in the peripheral blood eosinophilia and reticulocytosis without anemia. The bone marrow findings were characterized by increased erythropoietic activity and likewise eosinophilia. Despite a continuous loss of blood, an increased production in the bone marrow was sufficient to maintain the equilibrium between blood formation and blood loss. In the early stage of hookworm disease, real anemia may not develop if food and living conditions are adequate.

In the second type, they described these characteristics—anemia, microcytosis, hypochromia, eosinophilia, and reticulocytosis in the peripheral blood, and eosinophilia and marked erythropoietic activity of the bone marrow.

Hookworm anemia is hypochromic and microcytic, with a red cell count of 1-3.5 millions/c.mm. In advanced cases the red cell count may drop to below 1 million. Eosinophilia usually amounts to 5-15% although in more advanced cases eosinophilia may no longer be present. In some cases there may be a hypoplastic tendency of the bone marrow, but the condition is still reversible and the patient recovers.

The third type or irreversible type (aplastic anemia) is characterized clinically by severe anemia, dizziness, easy fatigability, and hemorrhagic diathesis. The erythropoietic activity of the bone marrow is almost nil. There is so-called panmyelophthisis with low cell count, relative lymphocytosis, and lack of erythropoietic, granulocytopoietic, and thrombocytopoietic activity of the bone marrow. In this stage antihelminthic and antianemic treatments are of no avail. Blood transfusions and iron are ineffective in improving the irreversible process. Aplastic anemia as a possible outcome of hookworm infestation has only rarely been mentioned. Stransky and Quintos observed 8 cases of aplastic anemia in hookworm disease. In one case there were hypochromia, anemia, leukopenia with granulocytopenia, relative lymphocytosis, thrombocytopenia, and reticulocytopenia in the peripheral blood. There were low cell count, relative lymphocytosis, poor erythropoietic activity, and megakaryocytopenia in the bone marrow. In 2 other cases although the outcome was the same as aplastic anemia, the hematologic picture at first was not definitely that of panmyelophthisis. One of these cases was an example of severe anemia with symptoms of early hemorrhagic diathesis. From the hematologic picture alone, the diagnosis of aplastic anemia could not be definitely made during the stay of the patient in the hospital for 7 weeks. However the outcome was fatal, and in 2 weeks after discharge, there was probably a rapid breakdown of the bone marrow. In another case, anemia was not extreme at first, with a count above 1 million, the bone marrow count was 105,000, which is about normal, and the differential count was not much altered, but the erythropoietic activity of the bone marrow was low, the ratio of nucleated red cells to white cells was 0.19:1.0 compared with 0.3:1.0; 0.6:1.0; and 1.1:1.0 in those that recovered in the second type. The hematologic symptoms, leukopenia with relative lymphocytosis, thrombocytopenia, low reticulocyte count, and absence of nucleated red cells in spite of the anemia shown by the peripheral blood, were in favor of aplastic anemia even in the beginning of the observation, despite the somewhat normal bone marrow findings. These authors believed that their cases of aplastic anemia were not simply coincidental with hookworm anemia but were actually related to it. They believed that the aplastic anemia of hookworm disease is due to bone marrow exhaustion following continuous blood loss in the presence of various factors, including dietary deficiency.

Pathophysiology of iron-deficiency anemia

The first response to iron loss is depletion of the iron stores, followed by an increase in plasma transferrin. The increase may represent an attempt to augment iron absorption. As this fails to take place, the serum iron falls so that an anemia is produced which at first is normocytic normochromic, but as the iron deficiency continues, it becomes microcytic hypochromic.

Differential diagnosis of iron-deficiency anemia

Anemias due to trace element deficiencies

The 2 trace elements of concern are cobalt and copper.

Cobalt: In man a diet devoid of cobalt should lead to the development of pernicious anemia since cobalt is a constituent of vitamin B_{12}. The completely cobalt-free diet is not feasible because of the ubiquity of cobalt. In animals cobalt deficiency diseases are well recognized and are frequently accompanied by microcytic anemia. In animal and man the administration of cobalt stimulates erythropoiesis and hemoglobin formation, probably by producing cellular anoxemia by interference with oxygen utilization through blocking of oxidation enzyme systems. Enzymatic blocking also accounts for the goiterogenic effect of high doses of cobalt.

Copper: Copper is a constituent of many essential enzymes, and together with iron it is necessary for the synthesis of hemoglobin. Milk is poor in copper so that a pure milk diet will lead to copper deficiency which is usually accompanied by a microcytic anemia. The hematologic action of copper may be linked to its effect on iron metabolism as it supports iron absorption and transfer. Red blood cells contain a copper-containing protein, erythrocuprein. The copper contents of red blood cells is in the range of the serum copper level, 93-114 mg./100 ml. The serum copper level may fluctuate, whereas the red blood cell copper level is usually constant. Copper is stored in the liver (higher in fetal liver), bones, and nerve tissue. Most of the serum copper is bound to an α-globulin, ceruloplasmin. Normal plasma contains 30 mg. ceruloplasmin/100 ml. A small amount of serum copper is loosely bound to albumin and is called direct reacting copper. It probably represents copper in transport. Plasma copper is elevated in liver diseases, pregnancy, tumors and schizophrenia and is decreased in hypothyroidism and nephrosis. In Wilson's disease, a form of hereditary deficiency of ceruloplasmin, the total serum copper level is normal or only slightly decreased because the direct acting copper is unusually high and thus makes up for the deficiency of ceruloplasmin.

Screening test for plasma ceruloplasmin[409a]

This method is based on the fact that ceruloplasmin catalyzes the oxidation of paraphenylenediamine, the rate of oxidation being proportional to the plasma concentration of ceruloplasmin. The oxidation results in a purple-blue compound. Prepared paper strips, etc., are available commercially.*

Anemia of infection

At first the anemia is usually normochromic normocytic in type, but after longer duration it becomes hypochromic. The serum iron level is low, as it is in true iron deficiency anemia, but the serum iron binding capacity is diminished, whereas in true iron-deficiency anemia it is increased. The serum copper is elevated (150-250 μg%), whereas in iron-deficiency anemia the serum copper is normal or only slightly elevated (130-150 μg%). The anemia responds poorly to oral or parenteral iron, and the iron tolerance test fails to raise the serum iron level, whereas in iron-deficiency anemia it is raised within a period of 2 hr. Korman[410] describes a differential test using intravenous radioiron containing 0.6 mg. carrier iron chelated with sodium ethylenediamine diortho hydroxyphenyl acetate (EDDHA) which allows differentiation of iron-deficiency anemia and the anemia of infection, renal disease, and depressed erythropoiesis.

The cause of the hypochromic anemia of infection is not clear. Heilmeyer[411] believes that the anemia is related, first, to a disturbance of the hemoglobin synthesis based on the dysproteinemia, and, second, to the activity of the reticuloendothelial system which mobilizes iron and deposits hemosiderin in the area of infection, thus increasing the tissue iron. There may also be an increase in the iron-containing enzymes so that the iron deposition can be considered a nonspecific defense mechanism. It should be recalled that anti-infectious properties have also been ascribed to transferrin. Radioiron studies show lowering and rapid transfer and turnover of the plasma iron as well as poor utilization of iron from hemoglobin synthesis. The red blood cell survival time is also shortened. The elevated serum iron level in acute hepatitis is an important exception to the phenomenon just described and constitutes a diagnostic test for this disease.

Sideroachrestic hypochromic anemias of Heilmeyer[412]

These anemias are due to interference with hemoglobin synthesis so that iron, though present in adequate amount, cannot be utilized. The serum iron level is high, and administration of iron fails to cure the anemia. The iron stores are loaded with iron (hemosiderin), and siderocytes and sideroblasts appear in bone marrow and blood.

Classification

- Essential hypochromic sideroachrestic anemia
  - Hereditary hypochromic sideroachrestic anemia
  - Refractory sideroblastic anemia (same as Dacie's refractory normoblastic anemia)
- Secondary sideroachrestic anemia
  - Anemia due to infection
  - Toxic anemia (lead)
  - Vitamin-deficiency anemia, pyridoxine deficiency
  - Thalassemia

Both essential hypochromic sideroachrestic anemias are due to enzymatic defects in the porphyrin metabolism. In the hereditary form there is a lack of enzymes which catalyze the conversion of coproporphyrin to protoporphyrin. In the acquired form there is a similar defect, but at the same time there is a defect in the enzyme which incorporates iron into the protoporphyrin ring.

In the secondary anemias the defect in iron utilization is only one aspect of a primary disease.

Pyridoxine (vitamin B_6)-deficiency anemia[413-415]

Pyridoxine-deficiency anemia is a hypochromic anemia characterized by high serum iron and high iron level in storage depots (hemosiderosis); it is refractive to treatment with Fe but responds to pyridoxine. The diagnosis can be made by the tryptophan tolerance test and by the response to treatment with pyridoxine. The tryptophan tolerance test consists of the estimation of xanthuric acid in the urine after an oral test dose of tryptophan.

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*Ortho Pharmaceutical Corp., Raritan, N. J.

Normally, tryptophan is converted to 3-oxykynurenin which gives rise to nicotinic acid with the help of intermediary compounds, enzymes, and vitamin B_6. If this pathway is blocked, oxytrynurenin gives rise to xanthuric acid. In vitamin B_6 deficiency under the conditions of the test, the xanthuric acid excretion is increased (normal, 50 mg.). Pyridoxine hydrochloride is utilized to form pyridoxal phosphate, a coenzyme involved in the synthesis of heme. Pyridoxine deficiency has been found in patients with malignant tumors, in children on a pyridoxine-deficient diet, in pregnant women, and in patients under treatment with isoniazid, a pyridoxine antagonist.

Thalassemia

Thalassemia is characterized by increased serum iron, presence of fetal hemoglobin, increased amount of hemoglobin-A_2, presence of target cells, basophilic stippling, nucleated red blood cells, hepatosplenomegaly, characteristic fascies, skeletal changes, and evidence of hemolysis. This disease is considered in the discussion of hemoglobinopathies.

MACROCYTIC ANEMIAS

There are 2 factors that must be considered in the discussion of pernicious anemia and related macrocytic anemias, namely, the intrinsic and the extrinsic antianemia factors. The intrinsic factor is an indefinite something which is neither hydrochloric acid, rennin, pepsin, nor lipase. It is deficient in pernicious anemia. It is found in the gastric secretion in other types of anemia, including aplastic anemia and chronic hypochromic anemia. The extrinsic factor, is present in beef muscle and in many other foods. It is not ether soluble, it passes through an ultrafilter, and it has been identified as vitamin B_{12} or cyanocobalamine, which chemically with its central cobalt surrounded by pyrrol rings somewhat resembles porphyrins. Macrocytic anemia occurs by reason of the lack of extrinsic factor in the diet or through a combination of dietary deficiency, insufficient intrinsic factor, or faulty absorption.

CLASSIFICATION

- Hereditary constitutional megaloblastic anemia
  - Pernicious anemia (absence of intrinsic factor)
- Symptomatic megaloblastic anemia
  - Malabsorption syndromes, vitamin B_{12} and folic acid deficiencies
    - Primary
      - Tropical sprue
      - Idiopathic steatorrhea
      - Celiac disease
    - Secondary
      - Regional ileitis
      - Small intestine resection
      - Blind loop operation
      - Chronic pancreatitis
      - Diverticula
      - Stomach resection
      - Gastric carcinoma
      - Hypothyroidism
  - Nutritional anemia, diminished supply of folic acid or vitamin B_{12}
    - Kwashiorkor
    - Goat milk anemia
    - Inadequate diet
  - Increased utilization of folic acid and vitamin B_{12}
    - Anemia of pregnancy
    - Anemia of infancy
    - Fish tapeworm *(Diphyllobothrium latum)* infestation
    - Pathologic intestinal flora in blind loop operations
    - Strictures of small bowel
    - Diverticula
    - Leukemia
  - Accompanying liver disease
  - Following administration of drugs
    - Anticonvulsants—Dilantin, primidone, amytal, Seconal, Rutonal, folic acid antagonists

HEREDITARY CONSTITUTIONAL MEGALOBLASTIC ANEMIA

Pernicious anemia

Pernicious anemia is known also as Biermer's disease or more properly morbus hunteraddisonii. It is clinically a symptom complex of a severe megalocytic anemia accompanied by marked intestinal symptoms, glossitis, achylia, dyspepsia and nervous symptoms, paresthesias, some cases showing the characteristic subacute combined degeneration of the cord and various psychic changes in the individual.

This disease was formerly always considered fatal: once the diagnosis was made, the death warrant was practically signed, but with the discovery of liver therapy by Whipple, Minot, and Murphy and the therapy of hogs' stomach by Castle, this disease has largely lost its terrors; its victims have been practically recalled from death to life.

Etiology

Pernicious anemia, as well as related macrocytic anemias, has been classified by Castle and Minot[406] as insufficient blood production due to nutritional deficiency of the bone marrow. Almost every type of macrocytic anemia showed prompt response to the administration of extracts of liver. If this liver therapy is discontinued, relapses occur. This seems to eliminate the former theories of infections, toxins, and noxae to explain the etiology of anemia of the macrocytic type. It was discovered in 1926 by Minot and Murphy[416] that liver therapy was effective in the treatment of pernicious anemia. This seemed to emphasize the deficiency theory of pernicious anemia. Minot, Murphy, and Stetson[417] proved that the reticulocyte response to the feeding of liver was a proof of the effectiveness of the agent. Castle[418] and others showed that untreated patients with pernicious anemia were deficient in gastric secretion of an essential thermolabile constituent or intrinsic factor. It was shown by Castle and Townsend[419] that the gastric secretion of the normal individual was ineffective by itself. Similarly, beef muscle was also ineffective on administration to suitable patients with pernicious anemia. The beef muscle, called the extrinsic factor, when combined with the normal gastric secretion, increased blood production and clinical improvement similar to that induced by liver therapy.

Pernicious anemia, therefore, develops in individuals whose gastric juice contains insufficient amounts or none of the intrinsic factor to react effectively with the food. Expressed in other terms, normal gastric juice with food produces the active principle which is stored in the liver, kidney, and other tissues of normal individuals and permits normal bone marrow function to go on and thereby prevents anemia. Castle and associates believe that pernicious anemia develops from a lack of the intrinsic or extrinsic factor or a little of each. They also believe that defects of intestinal absorption, even with a normal gastric mechanism, may lead to pernicious anemia. Sturgis and Isaacs[420] showed that pig stomach was effective in the treatment of pernicious anemia, and therefore, this organ had the same substance present as normal gastric juice of man. The liver substance or factor that helps the bone marrow to return to normal in cases of pernicious anemia is able to resist boiling, while that in normal human gastric juice and in pig stomach is rapidly destroyed by temperatures above 70° C. Meulengracht[421] proved that the factor is present in the pyloric region of the pig stomach.

The isolation of vitamin B_{12} and related cobaltamines appears to have solved the problem of extrinsic factor, with which it has come to be identified. Jukes[422] has summarized the biochemistry of folic acid and vitamin B_{12}. Vitamin B_{12} has a molecular weight of 1550-1750 and contains cobalt which accounts for its red color. It is effective in microscopic quantities in producing a remission in patients with pernicious anemia when given parenterally. Oral administration is ineffective unless administered simultaneously with or shortly after ingestion of intrinsic factor. If large doses of cyanocobalamine are administered by mouth, a small percentage is absorbed by diffusion without the help of the intrinsic factor.[424] A daily dose of 1000 μg will maintain normal concentration of cyanocobalamine in the serum.

Rubbo[423] presents the present-day concept of the enzyme systems involved in red blood cell production. He visualizes the action of folic (folinic) acid as catalyzing the pyrimidines from methionine. The second stage involves vitamin B_{12} as the catalyst for a combination of the pyrimidines and ribose (or desoxyribose) to form thymidine (nucleosides). Although a considerable amount of work has been done on vitamin B_{12}, showing its relation to nucleoprotein synthesis, there still remain many gaps in our knowledge of this complex mechanism. Assay of vitamin B_{12} activity has been carried on by determination of growths of *Lactobacillus leishmanii, Escherichia coli* mutants, *Euglena gracilis,*[425] and *Pateriochromonas stipitata.*

The mean normal serum value is about 350 $\mu\mu$g/ml. In pernicious anemia the value falls to 38 $\mu\mu$g/ml. In chronic myelogenous, leukemia, polycythemia, and in liver disease, high values are found, while in lymphatic leukemia and in pregnancy the values are normal or low. Microorganisms are able to synthesize B_{12}, and herbivorous animals are able to utilize vitamin B_{12} produced by intestinal bacteria. Man is unable to do so and has to rely on animal meat as source of vitamin B_{12}. Plants contain very little if any vitamin B_{12}, whereas liver, milk, cheese, fish, and molasses are rich in this vitamin. Vitamin B_{12} in food must be absorbed, and this is accomplished either with the help of intrinsic factor or by diffusion. In serum, vitamin B_{12} is bound to α-globulin.

Glass and associates,[426] using *Euglena gracilis* for assay, found oral administration of vitamin B_{12} together with a potent intrinsic factor concentrate caused a sharp rise in vitamin B_{12} blood level, but less than 0.5% in the urine. A rise in urinary output of vitamin B_{12} occurred after the reticulocyte peak.

Vitamin B_{12} has been linked with various isotopes for experimental studies which have yielded a considerable store of information, most of which has been directed at determining mechanisms involved in the interaction of the various factors involved in macrocytic anemias. Tests based on the low urinary output of orally ingested radioactive vitamin B_{12} have been used to detect pernicious anemia patients in remission.[427]

The attack on the nature of intrinsic factor continues unabated and the elusiveness of this factor continues to interest and baffle investigators. Castle[428] has reviewed the development of knowledge concerning the gastric intrinsic factor and its relation to pernicious anemia. He concludes, after a scholarly exposition with extensive bibliography, that he is "sure that there is now general agreement at least on the present state of uncertainty about the nature and function of the intrinsic factor."* He presents several theories, favoring the "catalyzed absorption theory" which assumes that intrinsic factor directly and specifically facilitates the absorption of vitamin B_{12} by the intestinal wall. According to Castle, pernicious anemia "would not develop if the patient could effect daily the transfer of a millionth of a gram of vitamin B_{12} the distance of a small fraction of a millimeter across the intestinal mucosa and into the blood stream. This he cannot do, principally as a result of failure of his stomach to secrete into its lumen some essential but unknown substance."*

In man the intrinsic factor is excreted by the gastric fundal glands and is probably a mucoprotein which has antigenic properties. It is protected from digestion by vitamin B_{12} and by food and consists of two components, one binding vitamin B_{12} and the other attaching itself to the intestinal mucosa. The latter component requires calcium ion, and therefore versenate and phytate inhibit vitamin B_{12} absorption. In addition there is some evidence that the intestinal mucosa contains a vitamin B_{12} acceptor which may become saturated by large doses of vitamin B_{12} and prevent further absorption. Vitamin B_{12} is stoichiometrically bound to intrinsic factor, is removed from food, and is absorbed mainly in the ileum. The intrinsic factor is probably absorbed with it. Vitamin B_{12} absorption is increased by the administration of sorbitol, thyroid, cortisone, and intrinsic factor if it is lacking and during pregnancy. Vitamin B_{12} absorption is decreased if intrinsic factor is absent, if intestinal pH is low, or if the intrinsic factor-vitamin B_{12} complex is used up prior to absorption. The speed of absorption depends on the amount of vitamin B_{12}. A small amount may take up to 4 hr., whereas massive amounts are rapidly absorbed. Much of the vitamin is stored in the liver and is liberated when the liver parenchyma is damaged. Other storage organs include the pancreas, spleen, heart, and kidney. In the kidney some of the vitamin B_{12} is probably reabsorbed. For vitamin B_{12} uptake by bone marrow a special serum factor may be necessary in addition to the intrinsic factor. Orally administered vitamin B_{12} is excreted in the bile, feces, and urine.[429, 430]

One of the fundamental problems in pernicious anemia is the question of why the intrinsic factor is diminished or absent. Pathologists have long been aware of the gastric atrophy found in pernicious anemia. From many statistical data one can get the impression that pernicious anemia is a familial disease characterized by varying degrees of gastric atrophy, which in some members of the family produces pernicious anemia but in the majority leads to clinically undetectable diminished intrinsic factor production. The latter are the potential pernicious anemia patients should factors appear which by their summation lead to vitamin B_{12} deficiency or to further intrinsic factor diminution, such as senile atrophy of the gastric mucosa, gastritis, inadequate diet, or hypothyroidism.

In the serum most of the absorbed vitamin B_{12} is bound to α-globulin, a complex called erythroglobulin. Free vitamin B_{12} is probably loosely attached to β-globulin. Vitamin B_{12} clearance studies show an increased binding capacity in myelogenous leukemia, whereas in lymphatic leukemia and in liver disease the binding capacity is normal.

Symptoms and signs

The symptoms of the disease are as follows. For a long period patients show a gradually increasing fatigue, with loss of appetite, vague symptoms of gastric disturbance achylic in nature, with burning of the tongue or about the gums. These patients at this time display the so-called Hunter tongue or Moeller glossitis, with rhagades about the mouth, gingivitis, marked dental caries, and extreme pallor. In these cases there is noted the light yellowing

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*From Castle, W. B.: New England J. Med. **249:** 603, 1953.

of the sclerae and other signs of possible hemolytic icterus. They show a golden yellow serum due to bilirubinemia as determined by the alcoholic (indirect) reaction of van den Bergh. Urobilinuria and urobilinogenuria are present.

There is usually complete achylia. The blood may or may not in the beginning show anemia, hyperchromia, and increase in size of red cells, whereas the hemoglobin and total red cell values persistently remain normal. One must emphasize at this point that the most significant indication of pernicious anemia is the **increase in the size of the red cells.**

Patients may develop nervous symptoms such as a numbness in the finger tips which is due to disturbances in the terminals of sensory nerves. They also have disturbances in taste and slight difficulty, too, with an otherwise good memory; they may show beginning impotency, perhaps slight unsteadiness in the gait, decreased or increased patellar reflexes, and Babinski, paralytic, or spastic ataxia.

There is another very remarkable phenomenon in pernicious anemia known as a remission—an unaccountable and sudden return to normal without any treatment.

The outcome of these cases is now favorable, but, as stated before, pernicious anemia was formerly considered a severe fatal anemia, preceded by a failing heart and edema of the lungs and subcutaneous tissues. Many of these patients developed the well-known funicular myelosis or subacute combined degeneration of the cord, a severe spastic or tabeslike status, complicated with decubitis and bladder and intestinal paralyses. Many patients showed preagonally hemorrhagic diatheses, at times definite retinal hemorrhages. Occasionally, a patient dies with symptoms that are highly suggestive of aplastic anemia.

Blood picture

The blood is watery, and consequently spreads are very thin. The total white cell count is low, usually around 4000/c.mm. The red cell count is low, 2,000,000-3,000,000/c.mm., sometimes as low as 320,000. The mean corpuscular hemoglobin (MCH) is high because of the disproportion between the count and the hemoglobin. The blood serum has the golden yellow appearance so well described by Naegeli, with the alcoholic (indirect), van den Bergh test **positive** and the (direct) test **negative.** There is in this disease a megalocytic blood picture with anisocytosis, poikilocytosis, and all degenerative forms of blood cells. The red cells, as just stated, are always definitely enlarged. One finds large hyperchromic cells, megalocytes. These cells have an increased cell volume and are completely filled with hemoglobin. The Price-Jones curve has a wide base, and the peak shows a displacement to the right. The red cells also show polychromasia, basophilic stippling, and Howell-Jolly bodies as well as nucleated forms. The reticulocyte count is low.

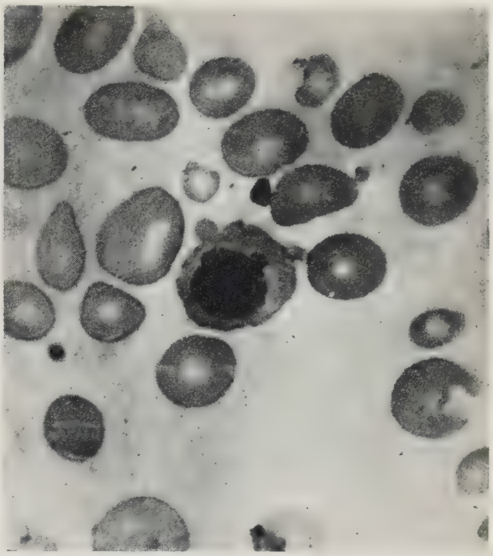
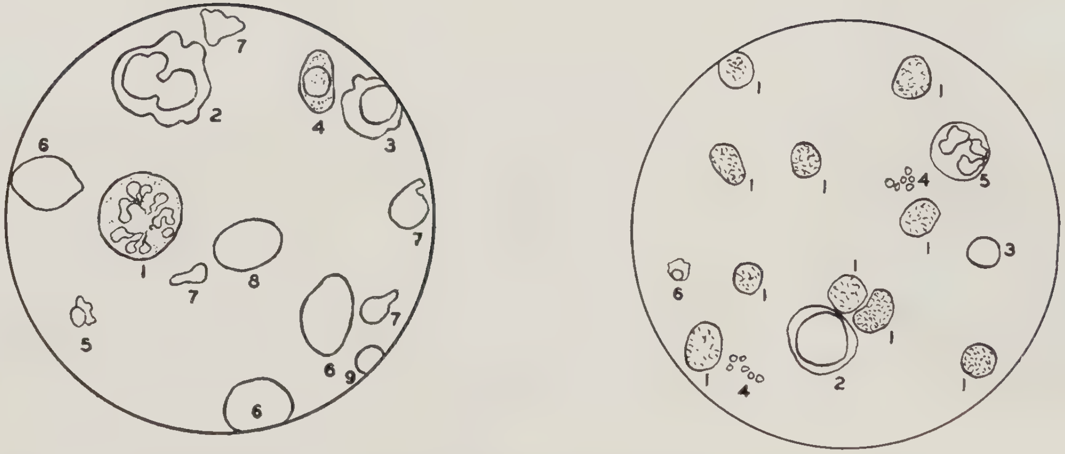


Fig. 421. Blood film of pernicious anemia showing hyperchromia, anisocytosis, poikilocytosis, macrocytosis, and a megaloblast. (×950.)

The white cells show neutropenia and lymphocytosis. **The nuclei of the neutrophils are highly segmented.** There are sometimes 6-8 segments to the cell, united to each other by fine stringlike connections. Arneth called this his characteristic right shift or **hypersegmentation.** These cells are also larger than normal and are called macropolycytes. The hypersegmentation is probably not a sign of old age but rather represents an abnormal configuration.

Blood platelets are absolutely decreased and are irregular in form and size. The entire blood picture has been called the **Naegeli syndrome.**

Blood crises are present in the beginning of the remissions of pernicious anemia or shortly before death. Here are seen many polychromatic and nucleated reds and also many regenerative leukocytes such as juvenile cells, myelocytes, promyelocytes, and even myeloblasts. The hemogram shown in Table 247 is from a blood crisis during pernicious anemia.



Advanced pernicious anemia

Before treatment

(Blood film; Giemsa stain; ×950)

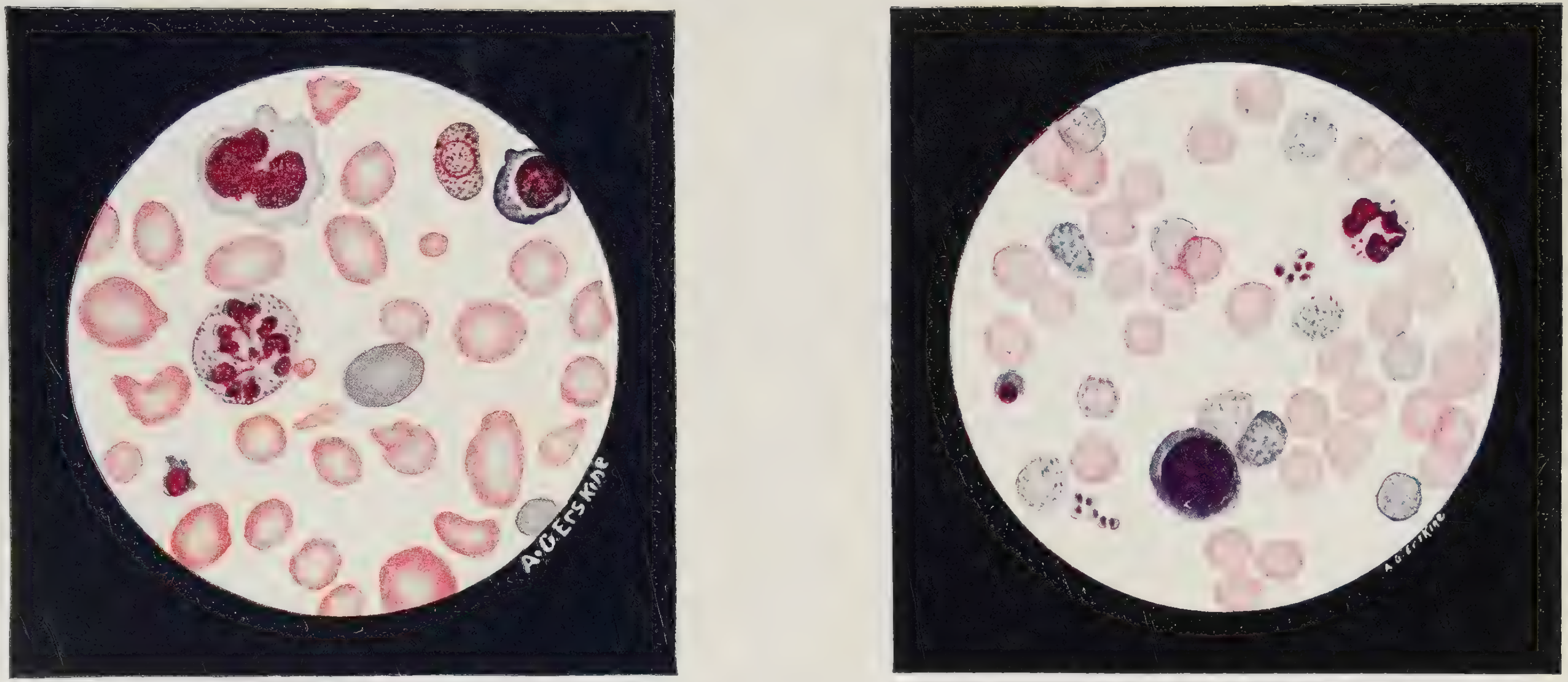
1. Giant hypersegmented neutrophil
2. Monocyte
3. Lymphocyte
4. Cabot ring body and basophilic punctation in a macrocyte
5. Large blood platelet
6. Megalocyte
7. Poikilocyte
8. Polychromatic megalocyte
9. Polychromatic normocyte

Note anisocytosis, poikilocytosis, polychromasia, basophilic punctation. Erythrocytes are macrocytic hyperchromic.

After therapy

(Brilliant cresyl blue; Giemsa stain; ×950)

1. Reticulocytes
2. Plasma cell
3. Polychromatic erythrocyte showing no reticulum
4. Blood platelets
5. Segmented neutrophil
6. Polychromatic microblast


A.G.Erskine
A.G.Erskine

Advanced pernicious anemia

Before treatment

After therapy

Fig. 422

Table 247

Basophils	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*
–	–	–	2	15	68	7	8

For every 100 leukocytes there were 1385 polychromatic normoblasts, 25 polychromatic macroblasts, 3 polychromatic megaloblasts, and 2 cells in mitosis.

There were marked karyorrhexis, coarse basophilic punctation, Howell-Jolly bodies, marked polychromasia, marked anisocytosis, and occasional myelocytes.

It is the custom to call a blood picture, in the absence of clinical symptoms, a **pernicious anemic blood picture** in reference to the above findings but not necessarily a case of **pernicious anemia.** It is interesting to note that from 3 to 4 days (maturation time of red blood cells) after the institution of liver therapy, there are marked polychromasia and a marked increase in the reticulocytes. The red cells return slowly to their normal form and color. The existing hemolysis ceases. There is also a rise in the hemoglobin, together with a rise in the number of red cells, but the rise of the red cells is faster than the rise of the hemoglobin, thus making the MCH fall. Leukocytes take on the aspect of hyperleukocytosis, often accompanied by eosinophilia.

Hypersegmentation of neutrophils: It has been known for a long time that there is a greater segmentation of the neutrophilic nucleus in pernicious anemia than in any other type of anemia. This was seen in the Arneth count when the number of cells with nuclei containing 3, 4, 5, or more segments is much higher than in normal blood. The increase in the cells of groups 4 and 5 of the Arneth count has been described by him as a shift to the right and is characteristic of the megaloblastic anemias. Some writers definitely state that this phenomenon is absolutely diagnostic of pernicious anemia, but it is difficult to agree with this statement in the light of the authoritative literature on the subject. In a monograph by Sturgis and Isaacs,[431] the following statement covers this question exceedingly well: "The polymorphonuclear neutrophils in pernicious anemia may have a greater percentage of nuclei with more lobes than normal; that is, the percentage of cells containing nuclei with 3, 4, 5, or more lobes is much greater in the blood of patients with pernicious anemia than in normal blood. This corresponds to what is described as a shift to the right in the Arneth count, and it occurs frequently when the anemia is severe. While some regard this as diagnostic of pernicious anemia, it is not characteristic of this condition alone as the same finding may be observed in other types of anemia. A shift to the left takes place during the early stages of the remission."*

Fleming[432] pointed out that the conditions in which a shift to the right occurs are pernicious anemia, sprue, and lymphocytic leukemia. Piney states that it occurs in certain cases of Hodgkin's disease prior to x-ray therapy. Davidson and Gulland[433] state that the cell bodies of the polymorphonuclear leukocytes are oval and not round in this condition; that this applies to both the large forms and those of ordinary size; further, that this change is most commonly found in severe cases and may affect all or nearly all the cells of that type to be seen in the blood film.

There are certain highlights in the diagnosis of this disease which must be emphasized.

1. Anemia often severe.
2. Diminution of blood platelets.
3. Erythrocytic changes, particularly increase in size.
4. Variations in size of the red cells.
5. Basophilic punctation, Cabot ring bodies, and polychromasia.
6. Occasional finding of megaloblasts.
7. Finding of normoblasts or nucleated red cells.
8. Neutropenia with high segmentation of the neutrophils, or Arneth's shift to the right.
9. Response of such cases to specific treatment.
10. Marked increase of reticulocytes first, after liver therapy, followed by a rise in the number of red cells before the hemoglobin begins to rise and consequent lowering of the MCH is highly suggestive of the accuracy of the diagnosis of morbus addisonii.
11. Macrocytosis should attract one's attention to this disease even before an anemia is noted.
12. A high or even a normal white count almost rules out this disease in cases of suspected anemia. In all cases one finds a lowered white blood count, sometimes down to 4000/c.mm.
13. It is claimed by Rous and Robertson[434] that the small red cells or microcytes with pointed tails, which may be called poikilocytes, are indicative of increased blood destruction.
14. Many basophilic punctated red cells are seen in this disease just as they are found in lead

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*From Sturgis, C. C., and Isaacs, R.: In Downey, H., editor: Handbook of hematology, New York, 1938, Paul B. Hoeber, Inc., vol. 3, p. 2251.

poisoning, but, as a rule, **polychromatic young red cells** are not found in Addison's anemia except at the onset of improvement of a treated case. It is for this reason that the count of the reticulocytes is very important.

15. There are very low red blood counts in this disease, sometimes as low as 500,000.
16. The white count is very low due to the failure of the bone marrow to produce neutrophils. In other words, the lymphocytes are not interfered with, whereas the neutrophils are lowered. Consequently, there is a picture of relative lymphocytosis. The higher the lymphocytosis, the severer is the prognosis, for the reason that the higher the lymphocytes, the more obvious it is that the bone marrow function for the manufacture of the neutrophils is partially destroyed.
17. As to the size of the red cells, the majority exceed 8.5μ in diameter.

The red cell survival time is shortened to ½ to ⅓ of the normal time. Pernicious anemia has a hemolytic component, and the red blood cells show increased hemolysis in hypotonic saline (incubated or using saponin). The plasma of patients with pernicious anemia also contains a hemolysin which acts on normal red blood cells transfused into untreated pernicious anemia patients. Because of the hemolytic component there is an increase in urobilinogen excretion and lowering of serum haptoglobin.

Reticulocyte response after erythrocyte maturation factor therapy: One of the most important phenomena in connection with the prognosis of this disease is the reticulocyte response after liver or other erythrocyte maturation factor therapy. In other words, after the use of these standard remedies, reticulocytes increase markedly in the peripheral blood. This is due to the normal maturation of the nucleated red blood cells in the bone marrow. This reticulocyte response does not show in the first 5 days of treatment for the reason that in this period the megaloblasts are developing into normal erythroblasts in the bone marrow. After the fifth day, there is a rapid increase in the number and percentage of reticulocytes. After the ninth to tenth day, there is a decrease in the percentage of reticulocytes, accompanied by an increase in the total number of red blood cells. From time to time thereafter there are increases in the percentage of reticulocytes due to the stimulation of blood formation. The greater the dose of the remedial agent used, the more rapid will be the reticulocyte response. This is dependent, too, upon the route of administration whether by mouth or by injection. The depth of the anemia in part determines the height of reticulocytosis; the more anemic the patient, the higher the reticulocyte response will be.

Bone marrow findings

There is marked (up to 10-30 times normal) erythroid hyperplasia, most of the cells belonging to a pathologic series, the megaloblastic series, which can be followed from the youngest promegaloblast to the more mature macronormoblast. These cells show nuclear-cytoplasmic asynchronism as well as a disturbance of the normal nuclear-cytoplasmic ratio. They crowd out most of the normal erythropoietic elements. The biochemical lesion responsible for the megaloblastic series is not known. Despite the fact that both folic acid and vitamin B_{12} are required for nucleoside synthesis, megaloblasts do not show a deficiency in desoxyribonucleic acid nor in ribonucleic acid. The marrow shows a high percentage of ineffective erythropoiesis. There appears to be an abnormal block or delay in the division of the erythroblasts while the division of proerythroblasts continues. Proerythroblastic cells acquire hemoglobin and retain their nuclei which fail to undergo pyknosis. The prime defect may be one of division rather than one of maturation. The delay in mitosis, the abnormality of cells, and the large number of ineffective cells lead to a marked erythroid hyperplasia which, despite its magnitude, is inadequate. Under the influence of antianemic therapy the megaloblasts begin normal division so that for a limited period (3 days) intermediate forms appear, and then finally normal erythropoietic elements can be found. The myelocytic elements are also involved in this process, and large myelocytes and giant metamyelocytes appear which are the result of abnormal division at the myelocytic level. These cells are apparently able to mature but are not able to divide. The abnormal leukocytic forms persist even after the erythroid series has returned to normal following therapy. Abnormal megakaryocytes as well as abnormal reticulum cells are noted.

Anatomically the principal change in the bone marrow in pernicious anemia is the replacement of the fatty marrow of the long bones with a soft red marrow which in the normal adult is restricted to the flat bones. Davidson and Gulland[435] found that the number of white cells in the bone marrow is greater than might be expected from the constant leukopenia present in the peripheral blood in these cases. Davidson and Gulland found also that the segmented neutrophils were reduced in number in the bone marrow and that the predominant type of leukocyte present was the myelocyte.

Nonhematologic findings

Examination of gastric juice: Examination of the gastric juice shows histamine-fast achylia. Stomach biopsy reveals atrophy of the mucosa. A very occasional case of true pernicious anemia may have free acid but will still lack intrinsic factor. This is usually seen in the rare pernicious anemia in childhood.[438] The occasional presence of hydrochloric acid and absence of gastric atrophy has led to a reappraisal of the gastric lesion in pernicious anemia with the result that Magnus[436] points out that the basic defect must be an inherited enzymatic defect of certain cells which prevents utilization of the intrinsic factor or absorption of vitamin B_{12}. It is this defect then which leads to atrophy of the gastric mucosa. According to Magnus[436] and Dameshek[437] this metabolic intrinsic cellular abnormality may at one stage be reversible or may go on to irreversible neoplasia. This process is seen in the cases of pernicious anemia which go on to polycythemia, leukemia, or cancer of the stomach.[439]

Another approach to diagnosis in the treated patient with pernicious anemia is the study of exfoliated material obtained by gastric lavage.[440] Rubin and Massey[441] have demonstrated a cell type in such washings which appears specific for pernicious anemia and which was found in 20 of 21 patients who had been under therapy for years. These cells are markedly enlarged atypical columnar epithelial cells with nuclei approaching twice normal size.

Examination of urine[450]**:** Olmstead and Hirschboeck[442] studied the fasting urorennin excretion in 50 patients with pernicious anemia and found that none of these patients excreted more than 0.5 rennin unit/10 ml. urine. In a control group aged 40-90 yr., 13 excreted less than 0.5 rennin unit, but the others excreted more than this. Their conclusions are that, if the morning urorennin excretion exceeds 0.5 rennin unit, it is likely that pernicious anemia is quite remote. On the other hand, excretions below 0.5 unit only imply the possibility of pernicious anemia but do not make a diagnosis.

Uropepsin determinations in urine show a similar fall. Urine amino acid assays reveal an increase in taurine. There is evidence that formiminoglutamic acid increases markedly in the urine of folic acid-deficient patients (from the normal of about 30 μg/ml. urine to as much as 275 μg/ml. urine). For assay methods of folic acid and formiminoglutamic acid see references.[442a, 442b, 451]

Examination of serum[449]**:** The serum findings include a low vitamin B_{12} content associated with an increased serum iron level and normal serum proteins. As in all hemolytic anemias, the haptoglobulin level is diminished. The prothrombin level may be low. Characteristically, the lactic dehydrogenase activity is about 10 times normal and may be related to the aforementioned extensive ineffective erythropoiesis in the bone marrow since megaloblasts are rich in this enzyme and probably release it when disintegrating.

Treatment

Folic acid therapy: Some patients show a sensitivity to liver—inflammation around the needle point after injection, together with a generalized itching sensitization of the skin. In some such cases, folic acid has been used. This is a vitamin which is known at this time to be very useful in the treatment of general macrocytic anemias, particularly nutritional anemia in the southern part of the United States and the tropics. It is highly useful in sprue, in pernicious anemia of pregnancy, in anemias associated with chronic alcoholism, and in carcinoma of the stomach.

It has been well established that folic acid will cause a complete hematologic response and will maintain blood levels in patients with pernicious anemia. However, the neurologic manifestations of this disease may continue to progress with folic acid therapy even though the hematologic response is satisfactory. For this reason, the use of folic acid **alone** is not recommended for the treatment of pernicious anemia.

Vitamin B_{12} therapy: In 1948 Smith[443] and Rickes and co-workers[444] isolated crystalline vitamin B_{12} from liver. It was found that infinitesimally small amounts of this substance were effective in restoring normal hematologic values in pernicious anemia patients in relapse. The dosage schedule is a minimum of 1 μg/day, but larger dosages are generally used. Blackburn[445] and Piney[446] have reviewed the modern treatment of pernicious anemia in excellent fashion.

A great deal of attention has been centered on the use of vitamin B_{12} orally, especially when combined with mucosal extracts of hog stomachs.[447] Small doses of vitamin B_{12} below 20 μg are ineffectual when given alone, but when vitamin B_{12} is combined with intrinsic factor containing mucinous materials from hog stomach, responses are optimal or suboptimal. Suboptimal dosages may mask symptoms until irreversible neurologic changes have taken place. The use of minimal dosages is decried because this alters the bone marrow picture sufficiently to make diagnosis impossible.

If there is no response to the administration of hog stomach and vitamin B_{12}, human gastric juice should be substituted. It is interesting that antibodies to human intrinsic factor have been shown to exist so that the possibility of an autoimmune type of pernicious anemia has to be considered.

Schilling[448] described a test for intrinsic factor in which he attempted to solve the dilemma of diagnosis in treated patients with pernicious anemia. The test consists of the administration of 0.5μc radioactive vitamin B_{12} (B_{12} Co^{60}) orally, followed in 2 hr. by 1000 μg of nonradioactive vitamin B_{12} parenterally. In normal subjects radioactive vitamin B_{12} appears in the urine within 4-6 hr. after the oral dose, and this excretion continues for 24 hr. In pernicious anemia, no radioactive substance appears in the urine.

SYMPTOMATIC MEGALOBLASTIC ANEMIAS

Malabsorption syndromes

Sprue*

Sprue has been described as an insidious chronic, remittent catarrhal inflammation of the alimentary canal occurring particularly in Europeans who are residing or have resided in tropical or subtropical climates. Undoubted cases of sprue have been reported in persons living in temperate climates who had not visited the tropics. These cases appear in the literature as nontropical sprue.

The disease is characterized by stomatitis and a bare, raw, painful tongue, recurrent attacks of cramps and diarrhea, voluminous, frothy, acid stools, loss of weight, and a severe anemia of the pernicious type.

The onset is usually insidious and manifests itself first by soreness of the buccal mucosa and vague digestive disturbances. The course is afebrile. The untreated patient gradually becomes weak, emaciated, irritable, and deadly pale.

The etiology is unknown. Only the concept of a deficiency disease has been able to withstand prolonged investigation. Castle, Rhoads, and collaborators, while working in Puerto Rico, confirmed the belief that deficient diets frequently antedate the onset of clinical sprue. Surely the disease is not contagious. The theory of the "monilia psilosis" as a causative agent has been discarded.

The disease is endemic in the East Indies, in South China, in India and Ceylon, in the West Indies, in Australia, in Japan, Korea, and the Philippine Islands. Its occasional occurrence in the United States is an accepted fact.

In a series of 150 cases studied in Puerto Rico, there were 121 caucasoids, 26 mulattoes, and only 3 Negroes. The disease appears to be, therefore, extremely rare in the Negro race. One third of the patients were less than 40 years of age, a point of difference from true addisonian anemia.

In general appearance some of the older patients presented strong resemblance to persons with pernicious anemia. Loss of subcutaneous fat and wrinkling and pigmentation of the skin, especially of the arms and legs, favor the diagnosis of sprue. In contrast to pernicious anemia the spleen was always normal or small. Although purpuric or scorbutic spots were occasionally found, retinal hemorrhages were uniformly absent.

The occurrence of osteoporosis and osteomalasia, reported as a common finding in nontropical sprue, was not found in the series of 150 cases already mentioned.

Investigations of the glucose tolerance showed uniformly a flat curve, favoring the theory of deficient intestinal absorption and making the hypothesis of a possible pancreatic disease less important in the understanding of sprue. Moreover the pancreas has been found normal in a high percentage of cases studied at autopsy.

The anemia in sprue is macrocytic, usually hyperchromic, and rarely hypochromic. The mean cell volume averages 123.6 c.μ. Nucleated red cells are very rarely found. Eosinophils are reduced. The white cell count is below normal, averaging 5280. See Table 248.

Studies of the aspirated sternal marrow yield interesting data. Megaloblasts are frequently seen. Usually there are more erythroblasts than normoblasts. Megakaryocytes, plasma cells, and monocytes are very rarely seen.

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*This discussion was contributed through the courtesy of Dr. Ramón M. Suárez, of the Hospital Mimiya, Santurce, Puerto Rico. The tables and the photomicrograph of the bone marrow were also furnished by Dr. Suárez. Dr. Suárez has had an unusual opportunity to study this disease in Puerto Rico.

Many of the erythroblasts are of the intermediate type. There is also a diminution in the amount of fat and in the myelogenous elements. Numerous Howell-Jolly bodies are seen in the bone marrow and peripheral blood supposedly due to the associated splenic atrophy.

Serum vitamin B_{12} levels are usually normal, whereas folic acid absorption is abnormal.

In Table 249 are shown the differential counts of the nucleated elements in the aspirated sternal marrow of sprue cases before treatment and again 10 days and 2 mo. following the institution of parenteral liver therapy. The erythroblastic elements, originally 41.73%, gradually diminished to 15.83%; the myeloblastic cells increased from 53.5% to 78.79%; the lymphoblastic elements in the

Table 248. *Hematologic data of 150 cases of sprue*

Sprue (150 cases)	*Lowest*	*Highest*	*Averages*
Hemoglobin	2.4 gm. (16.5%)	14.8 gm. (102%)	9.7 gm. (66%)
Erythrocytes	690,000	4,410,000	2,710,000
Packed cells	10.5 mm.	44 mm.	33.49 mm.
Leukocytes	1550	13,600	5280
Color index	0.64	2.2	1.22
Volume index	1.0	2.3	1.39
Mean cell vol.	89 c.μ	220 c.μ	123.6 c.μ
Mean cell Hb	26 μμg	59 μμg	36.6 μμg
Mean cell Hb (conc.)	20%	45%	26.1%

Table 249. *Bone marrow in sprue (averages)*

	Initial	*10 days*	*2 mo.*
Megaloblasts	6.31	1.79	0.60
Erythroblasts (early)	8.71	3.06	1.17
Erythroblasts (late)	11.12	5.23	2.80
Normoblasts	15.59	11.68	11.26
Myeloblasts	0.72	1.24	0.63
Promyelocytes	2.60	3.53	3.40
Myelocytes (neutrophils)	17.83	16.46	17.51
Myelocytes (eosinophils)	2.77	3.54	2.51
Myelocytes (basophils)	0.30	1.78	0.40
Polynuclears (neutrophils)	26.92	44.14	51.91
Polynuclears (eosinophils)	1.91	1.26	2.43
Monocytes	0.03	0	0
Lymphocytes	5.11	5.95	4.89
Megakaryocytes	0.07	0.24	0.06

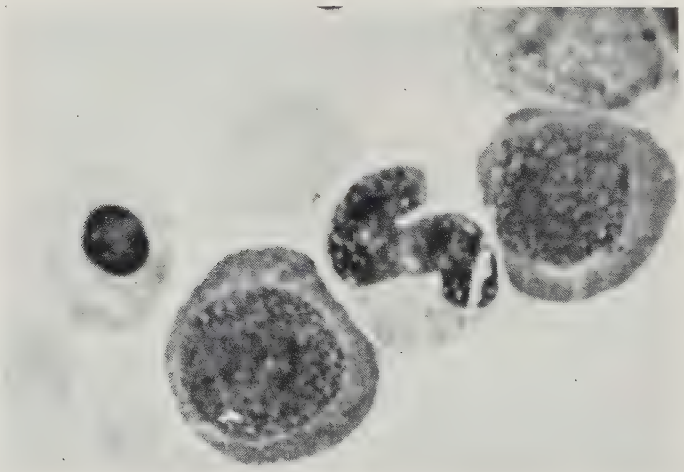


Fig. 423. Bone marrow showing megaloblasts, large normoblast, and neutrophilic juvenile cell. (Courtesy Dr. Ramón M. Suárez, Puerto Rico.)

marrow remained stationary during the observation period of 2 mo.

The van den Bergh test is the same in sprue as it is in pernicious anemia; i.e., the test points to an increased degree of blood destruction. There is approximately normal or slightly increased resistance of red cells to hemolysis by hypotonic sodium chloride solution, which is just about what is found in pernicious anemia. A reduction in blood platelets may occur, but it is variable and not to be depended upon as a diagnostic measure.

The most striking pathologic finding at autopsy is the almost total disappearance of the subcutaneous fat. The intestines are markedly thinned out, this atrophy especially affecting the mucosa. All internal organs are smaller, especially the liver and heart. Histopathologic changes of a chronic or ulcerative colitis were found in 30% of the patients.

Clinically, hematologically, and pathologically many of the cases of sprue are indistinguishable from pernicious anemia. Low blood calcium figures have been reported in sprue, which is not the case in pernicious anemia. Achlorhydria, an almost constant finding in pernicious anemia, is found in only 30% of sprue cases. Young children and adolescents are frequently affected with tropical sprue and very rarely if ever with pernicious anemia. Cord changes or neurologic manifestations, on the other hand, are extremely rare (about 2%) in sprue.

The 2 most important factors in the treatment of sprue are an appropriate diet and adequate liver or folic acid therapy. Ashford[452] insisted on the necessity of a high protein, low fat, and low carbohydrate diet. Liver extract should be administered parenterally. Both the diluted and the concentrated liver extracts have been found highly effective in all cases of sprue characterized by a macrocytic anemia, whether hyperchromic or hypochromic. Sprue is more resistant to treatment than is pernicious anemia and requires larger doses of liver, vitamin B_{12}, folic acid, and cortisone.

Megaloblastic anemia of Diphyllobothrium latum infestation

Discussion of anemia due to the *Diphyllobothrium latum* has been carried on in medical literature for some time. The question that has agitated the writers on this subject is whether this fish tapeworm causes the anemia which is so like a case of pernicious anemia or whether, as Magath[453] has stated, the presence of *Diphyllobothrium* is considered only as a trigger that sets off the illness. Schauman,[454, 455] the leading European investigator of this problem, maintains that *Diphyllobothrium* is capable of causing an anemia which is not distinguishable from true primary pernicious anemia, and that the reason the incidence of the disease is so small is that the "ordinary host is refractory toward the anemia-causing agent of the tapeworm." Piney[456] has called attention to the close resemblance between this anemia and true primary pernicious anemia. He believes that this condition may be elicited in some by the toxins of the fish tapeworm but thinks a constitutional predisposition probably exists in these patients. Birkeland[457] reviewed all the foreign as well as the American literature on *Diphyllobothrium* anemia, and after presenting the observations and opinions of apparently all who have written on this subject, in an unbiased but critical manner, concluded that the anemia occurring in symbiosis with an infestation by *Diphyllobothrium* is probably not due to any specific substance elaborated by this parasite but to a constitutional factor which would probably cause this type of patient to have a pernicious anemia without the presence of a parasite, granting that there occurs some unknown precipitating factor in this disease. In other words, he considers patients who suffer and survive *Diphyllobothrium* anemia as having had an abortive form of true primary pernicious anemia. Von Bonsdorff[458] suggests that the pernicious anemia picture of *Diphyllobothrium* infestation is due to two possible mechanisms. One involves the competition of parasite and host for vitamin B_{12}; in the event that the worm wins in this race, a deficiency develops in the host. An alternative or contributory mechanism is that intrinsic factor production may be diminished if the parasite is located high up in the small intestine. If the parasite moves lower down in the gastrointestinal tract, the pernicious anemia picture disappears.

Isaacs, Sturgis, and Smith studied a patient with a fish tapeworm infestation who had a typical blood and clinical picture of primary pernicious anemia which was amenable to liver therapy before removal of the tapeworm and who had no relapse of the anemia following the eradication of the parasite, even when liver therapy was stopped. Seyderhelm has demonstrated that the ground-up body of the fish tapeworm has a hemolytic action both in vitro and in vivo. Hunnicutt[459] has given a report on a very interesting case of a native of Finland who had a disease which was singularly like true pernicious anemia and cleared up under liver therapy and who later showed eggs and segments of worms. The diagnosis can be suspected when the characteristic anemia occurs in young persons in the presence of free hydrochloric acid, but it must be confirmed by examination of the stool.

In the megaloblastic anemias due to malabsorption are included the anemias that ensue from gastrectomy,[461, 462] blind loop operation,[460] regional ileitis,[460] etc. All these are believed to be due to altered bacterial flora (the achlorhydric stomach or gastric stump contains bacteria) which interferes with vitamin B_{12} absorption or utilizes vitamin B_{12} prior to absorption.

The diagnosis can be made in the presence of a suitable history and clinical findings and by a modification of the Schilling test. Addition of intrinsic factor does not improve absorption of radioactive vitamin B_{12}, but a course of tetracycline is followed by adequate absorption.

Megaloblastic anemia of pregnancy

The most common anemia in pregnancy is hypochromic in type, but in a large series reported, hyperchromic anemia occurred in 1 per 240 pregnant patients. Much higher incidence figures can be found in the recent literature, such as 1 in 67 or even higher. As a group, these patients are usually multiparous women who not only have a severe anemia but also a high incidence of premature births, hydramnios, and toxemia, conditions which lead to a high incidence of neonatal death (6%).

The anemia is frequently mixed (dimorphous). The megaloblasts are of the intermediate type, and there is evidence of iron deficiency. Serum vitamin B_{12} levels are normal. Intrinsic factor deficiency is not present, and folic acid absorption is normal. The cause of this type of anemia is not known. There is usually no evidence of malnutrition or of borderline nutrition which could be aggravated by pregnancy. The lowering of the serum vitamin B_{12} level in pregnancy is not related to the pathogenesis of this anemia. Folic acid treatment is curative.

Hormonal or metabolic disturbances have been suggested as being responsible for the anemia. Many years ago Vollmer and Gordan[463] showed a slight reduction of the red blood cell count of the female rat receiving pregnant mare's serum.

Megaloblastic anemia following medication (Dilantin)

According to Listberg and others,[464] most of the reports of megaloblastic anemia following anticonvulsant therapy appear in the British and Scandinavian literature. He reports one of the first cases in the American literature. The cause of the anemia appears to lie in an interference with the metabolism of folic acid and vitamin B_{12} similar to the one produced by folic acid antagonists. Antimalarial drugs such as pyrimethamine have also produced megaloblastic anemia after prolonged administration.

Other macrocytic and megaloblastic anemias

Megaloblastic or macrocytic anemias have been described in many conditions such as diseases of the liver, pancreas, and kidney, as well as in multiple myeloma, leukemia, polycythemia, and hemolytic anemias. The hyperchromic anemia accompanying hypothyroidism has long been recognized.

Folic acid acts as a coenzyme for a great number of intracellular metabolic reactions and is involved in the metabolism of thymine, purines, and pyrimidines, as well as uridylic acid (orotidylic acid) and folinic acid, all taking part in the formation of ribonucleic acid and desoxyribonucleic acid. There have been reports of megaloblastic anemias resulting apparently from metabolic defects involving such substances as folinic acid, ascorbic acid, uridylic acid, and thymine. The achrestic anemia described by Israels and Wilkinson may well fall into this group.

Macrocytic or megaloblastic anemia of hepatic cirrhosis

Severe liver disease may be accompanied by microcytic anemia, by simple macrocytosis without anemia, or by megaloblastic anemia.[466-468] Hypochromic anemia occurs only secondary to bleeding, whereas hemolytic anemia may be related to secondary hypersplenism. Various authors report the frequency of macrocytic and megaloblastic anemias as varying from 32-80%. The assumption that the degree of anemia is related to the degree of liver damage is not accepted by all authors. The bone marrow in cirrhosis is hyperplastic and shows erythroid hyperplasia, due mainly to an increase in normoblasts and macronormoblasts. Megaloblasts are rare, and the occurrence of true pernicious anemia in hepatic cirrhosis is doubtful, though reported by Rohr and others. Several etiologic factors can be mentioned for the occurrence of this anemia. The stomach may show alcoholic gastritis associated with depressed intrinsic factor content. The portal hypertension may lead to difficulty in absorption. The hypothesis that the liver parenchyma cannot fully accept, store, or release the antianemic factor cannot be supported, as liver extract obtained from cirrhotic livers converts the megaloblastic marrow of pernicious anemia to normal. On the other hand hepatic dysfunction leads to dysproteinemia, which in conjunction with hypovitaminosis may produce an alimentary type of macrocytic anemia. Bingham[467] divided the macrocytes into 3 types—thin, thick, and target. He felt that the thin macrocyte was specific for liver disease.

Endocrine macrocytic anemia

The anemia of hypothyroidism may be related to the frequent finding of gastric achylia in hypothyroidism, which also leads to some difficulties in vitamin B_{12} absorption.

NORMOCYTIC NORMOCHROMIC ANEMIAS

CLASSIFICATION

Hemolytic anemias
Aplastic or hypoplastic anemias
Myelophthisic anemia (myeloproliferative syndrome)
Anemia of pregnancy
Anemia of infection
Anemia of uremia
Anemia of endocrine disturbances
Anemia of avitaminosis

HEMOLYTIC ANEMIA

There are a number of blood dyscrasias which are grouped together as due to increased blood destruction. Paroxysmal hemoglobinuria, familial and acquired hemolytic jaundice, and sickle cell anemia are due to increased susceptibility of the red blood cells to destruction. There is a hemolysin present in the plasma in paroxysmal hemoglobinuria. In sickle cell anemia and hemolytic jaundice, in which there is a physical peculiarity of the erythrocytes, precise knowledge of its relation to increased blood destruction is lacking, although advances in the knowledge of "molecular disease of hemoglobin" has given us a new lead as to the basic disturbances.

All cases of hemolytic anemia have in common increased blood destruction and anemia. Hemolytic anemias may be divided into acute, subacute, and chronic types. The acute cases are due to malaria and other protozoa, bartonellae, bacterial toxins, chemical agents such as phenylhydrazine, saponin, trinitrotoluene, dinitrobenzol, aniline, amido and nitro compounds of phenol, benzol, toluol, lead, etc., snake venoms, extensive burns, and mismatched transfusions of isoagglutinogens A and B. All other types are those due to intragroup hemolytic transfusion reactions and hemolytic disease of the newborn. The chronic hemolytic anemias are classified as sickle cell anemia, chronic or acquired hemolytic jaundice, Cooley's anemia, and cases found with other chronic diseases such as pernicious anemia, Hodgkin's disease, and carcinomatosis.

Classification

Based on the foregoing, hemolytic anemias may be classified according to their clinical course into (1) acute, (2) subacute, and (3) chronic.

On the basis of etiology they may be classified into anemias due to intracorpuscular defects and those due to extracorpuscular defects.

- Intracorpuscular defects
  - Hereditary spherocytosis
  - Enzyme-deficiency hemolytic anemias
  - Hereditary (congenital) nonspherocytic hemolytic anemia
  - Other congenital hemolytic anemias (Heinz body)
  - Thalassemia
  - Paroxysmal nocturnal hemoglobinuria and paroxysmal cold hemoglobinuria
  - Hemoglobinopathies
  - Hereditary elliptocytosis (ovalocytosis)
  - Pernicious anemia
- Extracorpuscular defects
  - Antibodies: isoimmune and autoimmune
  - Infections: bacterial, viral, and parasitic
  - Toxins: snake venom
  - Physical agents: burns
  - Hypersplenism

Features of all hemolytic anemias and methods of investigation

Red cell survival time

The common denominator of all hemolytic anemias is the increased rate of blood destruction and the shortened life span of red blood cells. The normal life span of the red blood cell is 120 days plus or minus 20 days.

Ashby survival technic: The Ashby technic of differential agglutination requires the administration of compatible donor cells which nevertheless differ in type from those of the recipient. O cells for instance can be given

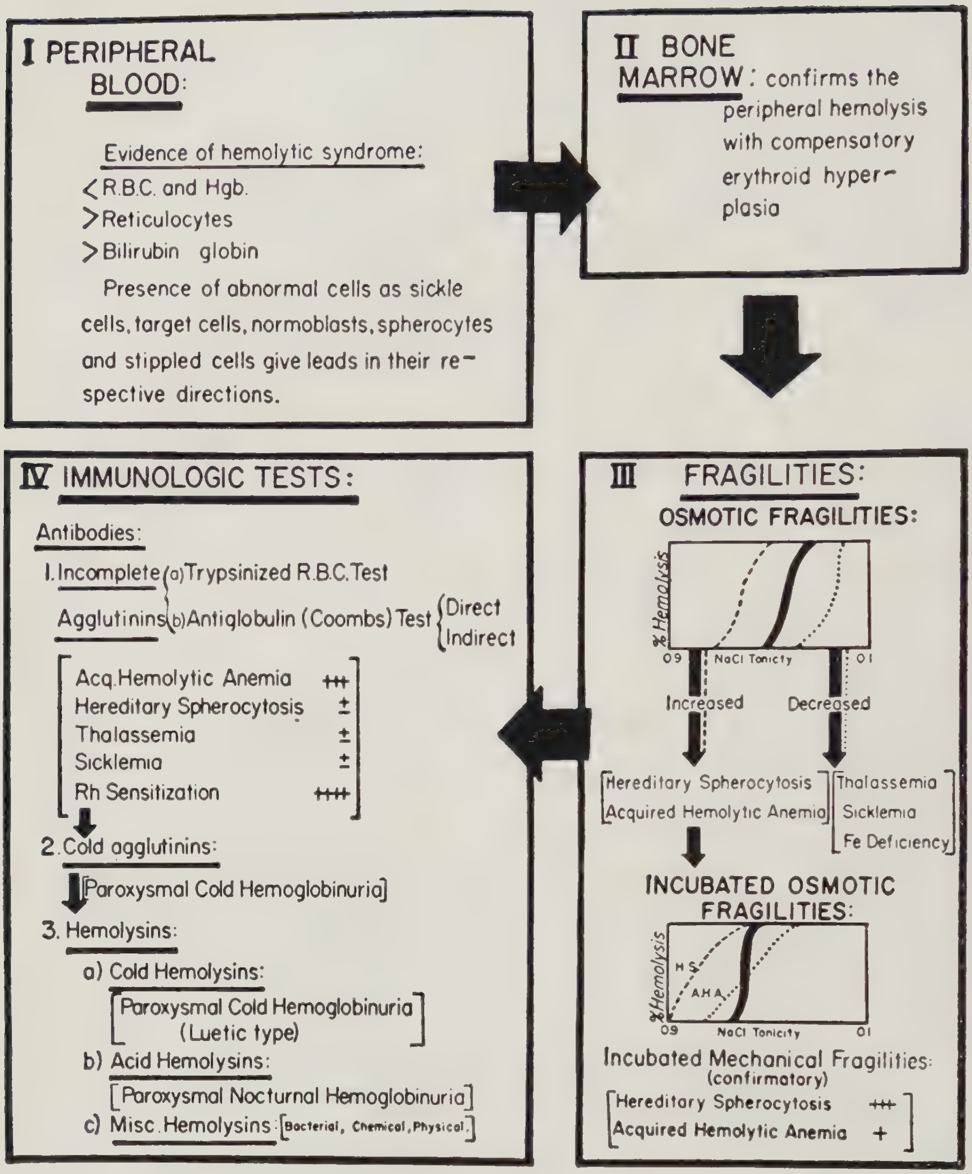

I PERIPHERAL BLOOD:
Evidence of hemolytic syndrome:
< R.B.C. and Hgb.
> Reticulocytes
> Bilirubin globin
Presence of abnormal cells as sickle cells, target cells, normoblasts, spherocytes and stippled cells give leads in their respective directions.
II BONE MARROW: confirms the peripheral hemolysis with compensatory erythroid hyperplasia
III FRAGILITIES:
OSMOTIC FRAGILITIES:
% Hemolysis
0.9
NaCl Tonicity
0.1
Increased
Decreased
Hereditary Spherocytosis
Acquired Hemolytic Anemia
Thalassemia
Sicklemia
Fe Deficiency
INCUBATED OSMOTIC FRAGILITIES:
% Hemolysis
H.S.
A.H.A.
0.9
NaCl Tonicity
0.1
Incubated Mechanical Fragilities: (confirmatory)
Hereditary Spherocytosis +++
Acquired Hemolytic Anemia +
IV IMMUNOLOGIC TESTS:
Antibodies:
1. Incomplete Agglutinins
a) Trypsinized R.B.C. Test
b) Antiglobulin (Coombs) Test
Direct
Indirect
Acq. Hemolytic Anemia +++
Hereditary Spherocytosis ±
Thalassemia ±
Sicklemia ±
Rh Sensitization ++++
2. Cold agglutinins:
Paroxysmal Cold Hemoglobinuria
3. Hemolysins:
a) Cold Hemolysins:
Paroxysmal Cold Hemoglobinuria (Luetic type)
b) Acid Hemolysins:
Paroxysmal Nocturnal Hemoglobinuria
c) Misc. Hemolysins: Bacterial, Chemical, Physical.

Fig. 424. Hemolytic syndromes—laboratory differentiation. Peripheral blood (I) and the marrow (II) indicate a hemolytic process. Osmotic fragilities (III) differentiate hereditary spherocytosis and acquired hemolytic anemia, on the one hand, from thalassemia, sicklemia, and iron-deficiency anemias on the other. Hereditary spherocytosis (H.S.) and acquired hemolytic anemia (A.H.A.) are differentiated from each other by incubated osmotic and mechanical fragility tests. Cells from hereditary spherocytosis show a pronounced increase in fragility (broken line). Cells from acquired hemolytic anemia show less change and usually assume an asymmetric curve (dotted line) which may cross over the normal incubated control. If the diagnosis is still not evident, immunologic tests (IV) are done. (From Wright, Claude-Starr: Mod. Med. 22:78, 1954.)

to A, B, and AB recipients or M and N cells to N and M recipients. Despite the obvious serologic difficulties reliable results can be obtained if blood samples of the recipient are tested for survival of transfused cells by differential agglutination at regular intervals (daily to every third or fifth day).

Red cell survival time measured by isotopes: Glycine labeled with N^{15} can be used to measure survival time in vivo as it is incorporated into the heme of newly formed red blood cells. By this method it has been shown that the destruction of normal red blood cells is a function of their age. C^{14} can also be used to tag glycine. Another in vivo method of tagging utilizes Fe^{59}. $Na_2Cr^{51}O_4$ and Fe^{59} are employed to label a patient's own cells in vitro after incubation and these are then reinjected into the patient. Since Fe^{59} is reutilized after the destruction of red blood cells, the use of Cr^{51} is the preferable method. The isotope methods allow the measurement of the survival time of the patient's own cells in his own circulation as well as the localization of the site or sites of their destruction.

The tagged cells allow 3 methods of investigation: (1) injection of the patient's own tagged cells into himself, (2) injection of the patient's tagged cells into a normal compatible recipient, and (3) injection of compatible normal donor tagged cells into the patient.

In hemolytic anemias characterized by an intrinsic defect, methods 1 and 2 will show a shortened survival time, whereas method 3 will result in a normal survival time. The defect can thus be localized in the patient's own red blood cells, which are rapidly destroyed in a normal recipient.

In hemolytic anemias due to an extrinsic defect, methods 1 and 3 will result in a shortened survival time, whereas method 2 will give a normal survival curve. The defect thus lies in the patient's circulation which rapidly destroys normal donor cells.

In a combined defect, that is extrinsic and intrinsic, both methods 2 and 3 will exhibit shortened steep curves as does, of course, method 1.

Hemolysis

There are 2 major methods of red cell destruction: (1) extravascular hemolysis and (2) intravascular hemolysis.

Extravascular hemolysis

In the aged cell, certain enzymatic changes[470] take place which lead to a decrease in adenosine triphosphate and coenzyme I, a process which ushers in the death of the cell. These cells are then engulfed by the macrophages of the reticuloendothelial system of the bone marrow, spleen, liver, lung, etc. and are destroyed so that their hemoglobin is freed. The iron is rapidly reutilized and is carried to depot organs by transferrin, whereas the porphyrin moiety is converted into free nonconjugated bilirubin. In the liver the latter is conjugated to glucuronic acid and is then excreted as conjugated bilirubin, which in the bowel is converted by the action of bacteria into urobilinogen and is excreted in the feces and urine.

Laboratory investigation of extravascular hemolysis

Schematic pathway of pigment

Extravascular breakdown → increased serum bilirubin → icterus → increased urine and fecal urobilinogen

Serum bilirubin

The indirect van den Bergh value is increased due to the presence of free (nonconjugated) bilirubin. The serum bilirubin depends on 3 factors: (1) rate of production, (2) liver function, i.e., its ability to conjugate, and (3) number of circulating red blood cells. An increase in the bilirubin level of more than 3 mg./day is evidence of increased blood destruction.

In newborn infants the liver is unable to conjugate bilirubin for several days, and therefore all bilirubin is indirect reacting.

Urobilinogen

Fecal urobilinogen: This is a colored pigment resulting from reduction of bilirubin. It depends not only on the rate of hemoglobin breakdown but also on the total red blood cell count and the total circulating hemoglobin. The hemolytic index represents the correlation of fecal urobilinogen to the total circulating hemoglobin.

$$\text{The hemolytic index} = \frac{\text{Fecal urobilinogen (mg./day)} \times 100}{\text{Total circulating Hb (gm.)}}$$

Normal: 100-200 mg./day.

Urinary urobilinogen: Urobilinogen is absorbed from the bowel into the blood stream but is excreted by the kidneys into the urine only if the plasma level exceeds the amount the liver is able to reconvert into bilirubin. The level of the urinary urobilinogen is, therefore, more a test of liver function than an indicator of increased hemolysis. Urinary urobilinogen on standing is converted into a dark pigment, urobilin.

Normal: 0-4 mg./day or production of a red color with Ehrlich aldehyde reagent in a 1:20 dilution.

Daily hemoglobin breakdown: 6 μg/day.

Intravascular hemolysis

Normally a small amount of blood is destroyed within the blood stream. The liberated hemoglobin is bound to plasma proteins called haptoglobins, which have a special affinity for hemoglobin. The haptoglobin-hemoglobin molecule is taken up by the reticuloendothelial system which splits the iron off the hemoglobin molecule and degrades the protein. The haptoglobin removed from the circulation is only slowly replaced (10 days) so that future waves of hemoglobin may find only a small amount of haptoglobin to bind and will thus be excreted by the kidney at a much lower serum hemoglobin level than is normally the case. If the free hemoglobin exceeds 140 mg./100 ml., the haptoglobin binding capacity is saturated so that free hemoglobin appears in the serum (**hemoglobinemia**) and in the urine (**hemoglobinuria**). The renal threshold for hemoglobin depends on the level of plasma haptoglobin[472-474] and on the tubular reabsorption which removes hemoglobin from the glomerular filtrate, creating a certain threshold level. If the haptoglobin level is low, as it is in newborn babies and some adults (Negroes) and in chronic hemolytic states, the renal threshold appears to be lowered, as free hemoglobin is excreted at relatively low plasma levels. In contrast with free hemoglobin, the hemoglobin-haptoglobin complex is not excreted by the kidneys. Some of the free hemoglobin is also taken up by the reticuloendothelial system and is converted to hemosiderin. Bruce and Lewis[471] have shown that disappearance of haptoglobin follows hemoglobin turnover of twice the normal rate.

Haptoglobin levels are raised in certain neoplasms with metastases, in infections, in Hodgkin's disease, in obstructive jaundice, and after steroid therapy so that the effect of hemolysis may be masked. A decrease in haptoglobin occurs in hemolysis whether extravascular or intravascular in nature, in liver disease, in infectious mononucleosis, and in congenital absence of haptoglobins.

Genetic studies have shown that 4 phenotypes of haptoglobin occur which are controlled by a pair of allelomorphic genes. The occurrence of ahaptoglobinemia may be due to this suppression effect.

Methods for determination of serum haptoglobin[475]

The following methods for the determination of serum haptoglobin are available: paper electrophoresis, titrimetric analysis, starch gel electrophoresis, immunoelectrophoresis, and spectrophotometry.

Paper electrophoresis[476]*—Nyman*

This method is based on the fact that when serum containing free hemoglobin and haptohemoglobin molecules is subjected to electrophoresis and then stained for peroxidase activity, 2 bands are seen. The band further away from the application point represents the hemoglobin-haptoglobin molecules, whereas the band closest to the application point represents free hemoglobin. A series of test tubes is prepared, each containing 0.1 ml. serum. To these test tubes hemoglobin solutions increasing in concentration by increments of 10 mg.% are added. These mixtures are then electrophorized and stained for peroxidase activity. The last strip which shows only one band of the hemoglobin-haptoglobin complex represents the highest hemoglobin concentration in which the entire hemoglobin is taken up by haptoglobin. The concentration of haptoglobin in the serum is expressed as the mg.% hemoglobin the serum is capable of binding.

Titrimetric analysis

Two methods are available, one in which an excess of hemoglobin is added to the constant amount of unknown serum and the other in which increasing amounts of the unknown serum are added to a constant amount of hemoglobin. In each case the hemoglobin binding capacity of the serum is measured and expressed in mg.

Starch gel electrophoresis and immunoelectrophoresis

These methods are useful in developing the genetic types of haptoglobin.

Spectrophotometric method for determination of serum haptoglobin—Owen, Better, and Hoban

The method presented here is based on the peroxidase activity of haptoglobin-methemoglobin complexes. Diluted serum is incubated with a standard methemoglobin solution to permit formation of a methemoglobin-haptoglobin complex. A buffered guaiacol solution and peroxide are then added, and this mixture is incubated in the dark. During this incubation, in the presence of peroxidase activity, the guaiacol is oxidized to a colored compound. The amount of this colored compound is determined spectrophotometrically. The amount of guaiacol oxidized, corrected for its nonenzymatic oxidation, is a measure of the peroxidase activity of the haptoglobin-hemoglobin complex and in turn of the haptoglobin content of the serum.

Reagents:

1. Guaiacol reagent.
   - (a) Dissolve 3.72 gm. guaiacol in about 700 ml. distilled water.
   - (b) Add 100 ml. 1M acetic acid.
   - (c) Adjust the pH of this solution to 4.0 by the addition of 1M sodium hydroxide and dilute to final vol. 1 L.

Alternately, the guaiacol may be dissolved in 0.1M sodium acetate buffer, pH 4.0, to a final volume of 1 L.

2. 0.05M Hydrogen peroxide.
   - (a) Prepare immediately before use by diluting a stock solution which has been previously standardized by titration against standard potassium permanganate. Commercial hydrogen peroxide is usually supplied as 30% solutions that are approximately 10M.
3. 0.15M Sodium chloride.
4. Methemoglobin solution, 50 mg./100 ml.
   - (a) Collect the erythrocytes from 30 ml. human blood by centrifugation and wash 3 times with 0.15M saline and measure the final packed volume.
   - (b) To 8 vol. these packed cells, add 3 vol. water and 1 vol. ether.
   - (c) Shake this mixture to effect hemolysis of the cells, centrifuge, and pipet off the hemolysate.
   - (d) Determine the concentration of hemoglobin in the hemolysate and dilute the hemolysate until the hemoglobin concentration is exactly 1 gm./100 ml.
   - (e) To 25 ml. of this solution add 10 ml. potassium ferricyanide (100 mg./ml.) to convert hemoglobin to methemoglobin.
   - (f) Allow to stand 10 min.
   - (g) Dilute to 500 ml., yielding a final solution with a methemoglobin concentration of 50 mg./100 ml.

This solution may be stored for several weeks at 0-2° C.

Procedure:

1. Place 2 test tubes, each containing 5 ml. guaiacol reagent, in a water bath fitted with a lid and set at 30° C. At the same time place in the bath a tube containing sufficient hydrogen peroxide solution for the tests to be performed.
2. Add 1 ml. serum to be tested to 4 ml. saline. To 2 ml. aliquots diluted serum, add 1 ml. methemoglobin solution to one and 1 ml. water to the other which is to serve as a reagent blank and incubate 10 min. at room temperature.
3. Add to one of the tubes containing guaiacol reagent 0.1 ml. serum-methemoglobin solution and to the other 0.1 ml. serum-water solution. Immediately add to each 1 ml. hydrogen peroxide solution, mix thoroughly, and replace in the covered bath.
4. Incubate 8 min. remove from the bath, and determine the optical density of each at a wavelength of 470 mμ using a water blank to set the instrument to zero optical density.

Subtract the optical density of the reagent blank from that of the test and refer this corrected OD to a calibration curve to obtain serum haptoglobin concentration.

Express the concentration of haptoglobin in terms of its methemoglobin binding capacity.

Although no cuvet size is specified, the conditions described should be satisfactory for use with cuvets supplied with most commercial photometers.

If readings too high or too low are obtained, the test should be repeated, using either a higher or lower dilution of serum.

Calibration curve:

1. Pipet 1 ml. aliquot methemoglobin solution into each of 11 tubes.
2. Add to these respectively increasing amounts of a pooled normal serum free from hemoglobin. The first tube receives 0, the second 0.1, then 0.2, 0.3, 0.4, on to the final tube which receives 1 ml.
3. Adjust the volume in each tube to 2 ml. with saline.
4. Add aliquots of 0.1 ml. each tube to 5 ml. guaiacol and prepare a blank tube containing 0.1 ml. of saline and 5 ml. guaiacol.

Submit these to the procedure described for the unknown beginning with step 3.

Set the spectrophotometer to zero OD with the blank and obtain the OD of each of the tubes.

Plot these readings against the amounts of serum in each reaction tube. The optical density of each successive tube will increase proportionately until a maximum value is reached, after which successive increments of serum will result in no increase in optical density. Thus will be obtained a straight line curve of optical density versus serum amount which will have a sharp inflection point and then level off. The point of inflection and failure to continue to increase indicates that all of the methemoglobin present in the reaction tube has been bound by the haptoglobin in the serum added. This point then represents the equivalent of a serum containing haptoglobin having a binding capacity of 250 mg. methemoglobin/100 ml. The sloping part of the curve relates lower readings to the corresponding haptoglobin contents.

For example, if the point of inflection is reached in the reaction tube containing 0.025 ml. serum, then this OD corresponds to 250 mg./100 ml. binding capacity. The tubes containing smaller amounts of serum then contain 0.005, 0.010, 0.015, and

0.020 ml. serum and correspond, respectively, to 50, 100, 150, and 200 mg./100 ml. methemoglobin binding capacity.

Discussion: Under the conditions chosen here there are a number of contributors to the total oxidation of guaiacol in addition to haptoglobin-methemoglobin complex. These include some nonenzymatic oxidation and the peroxidase activity of the serum itself, which are compensated for by the use of a serum blank, and the peroxidase activity of unbound methemoglobin, which is compensated for by the method of calibration.

The sensitivity of the guaiacol reagent changes with time; therefore it is essential that the calibration curve be repeated at frequent intervals. It is also essential that a covered water bath be used and that readings be made immediately after removal of samples from the bath, as the color formed during the reaction fades significantly on exposure to daylight.

Calibration of haptoglobin content in terms of its methemoglobin binding capacity is chosen since it is possible to measure hemoglobin with reasonable precision, and at present no source of purified haptoglobin exists.

The **mean normal serum haptoglobin content** has been described by a number of authors to be equal to a binding capacity of approximately 100 mg./100 ml.

Laboratory investigation of intravascular hemolysis

Schematic pathway of pigment

Red blood cell → hemoglobin (hemoglobinemia) → haptoglobin → methemoglobin → methemalbumin → hemoglobinuria → methemoglobinuria → hemosiderinuria.

Hemoglobinemia

Color of serum is pink. The test for peroxidase activity of hemoglobin is positive. Spectroscopic examination of plasma reveals free hemoglobin.

Hemoglobin determination in plasma—benzidine method of Crosby-Furth[476a]

Principle: The reaction between benzidine, hydrogen peroxide, and heme-containing pigments produces first a green color, later a blue color, and finally a violet color.

Glassware: Acid cleaned.

Reagents:

1. Benzidine reagent, 1%.

Benzidine base . . .	1 gm.
Glacial acetic acid . .	90 ml.
Distilled water . . .	up to 100 ml.

Keep in refrigerator.

2. Hydrogen peroxide, 1%.
   (a) Prepare by dilution from 30% or 3% hydrogen peroxide. Prepare fresh daily.
3. Standard hemoglobin solution.
   (a) Wash 2 ml. packed red cells 3 times with 0.9% saline.
   (b) Lyse by repeated freezing and thawing, add an equal vol. saline, and centrifuge at high speed to remove ghosts.
   (c) Aspirate the supernatant solution to a graduated tube.
   (d) Determine its hemoglobin concentration by the method in use in the laboratory and dilute with saline to a concentration of 10 gm. Hb/100 ml. This stock standard may be stored frozen for several months. The working standard is prepared by diluting 0.02 ml. stock in 10 ml. saline. This standard contains 20 mg. Hb/100 ml.
4. Diluent solution.
   (a) Dilute 10 ml. glacial acetic acid to 100 ml. with distilled water

Water for reagents must be free of sulfate; test with $BaCL_2$.

Procedure:

1. Withdrawal of blood. Wet a 5 ml. syringe with heparin and expel excess. Make venipuncture in usual manner and permit syringe to fill by venous pressure alone or if necessary use very gentle suction. Mix blood in the syringe by gently inverting several times, transfer to tube, and centrifuge at 1000 r.p.m. for 10 min. Aspirate the upper half only of the plasma layer and recentrifuge. Hemolysis must be avoided.
2. Measure 1 ml. benzidine solution into each of 3 tubes. Add, respectively, 0.02 ml. unknown plasma, 0.02 ml. standard hemoglobin solution (20 mg. Hb/100 ml.), and 0.02 ml. saline.
3. Add 1 ml. peroxide to each tube and mix immediately. Allow to stand 20-30 min. for color development.
4. Add 10 ml. diluent solution to each tube, mix by inversion, and allow to stand 10 min.
5. Determine the optical density (OD) of standard and unknown at 515 mμ, using the reagent blank to set the spectrophotometer to zero OD.

Calculation:

$$\frac{\text{D unknown}}{\text{D standard}} \times 20 = \text{mg. Hb/100 ml. plasma}$$

Comments: The sensitivity of the method as described is such that with most spectrophotometers concentrations of Hb as high as 40 mg./100 ml. may be determined satisfactorily. If OD values greater than 0.6 are obtained, the plasma may be diluted prior to analysis and the result corrected for this dilution. If greater accuracy is desired in the lower range, 0.05 ml. plasma may be used; again the result must be corrected for the difference in the volume of plasma used.

Serum haptoglobin

In the absence of the interfering factors previously discussed, the haptoglobin level is a sensitive test for hemolysis. It is decreased in hemolysis.

Hemoglobinuria

Tests for hemoglobin such as guaiac or benzidine are positive in the absence of red cells in the sediment

Methemalbuminemia

On spectroscopic examination the band is rather faint.

Schumm test:

1. Cover serum or plasma with ether and add 0.1 vol. concentrated ammonium sulfide.

In the presence of methemalbumin a positive Schumm test will show a sharp band at 558 mμ.

Hemosiderinuria

This results from both extravascular and intravascular hemolysis and gives a positive Prussian blue test of the urinary sediment (renal cells and often casts).

Hematologic findings in hemolytic anemia

Peripheral blood:

Anemia: There are decreased hemoglobin and red cell values. The anemia is usually normochromic.

Spherocytosis: Spherocytes occur in both acquired and congenital hemolytic anemia. They represent a prehemolytic state, being easily hemolyzed in hypotonic saline solution (for details, see previous discussion of spherocytes).

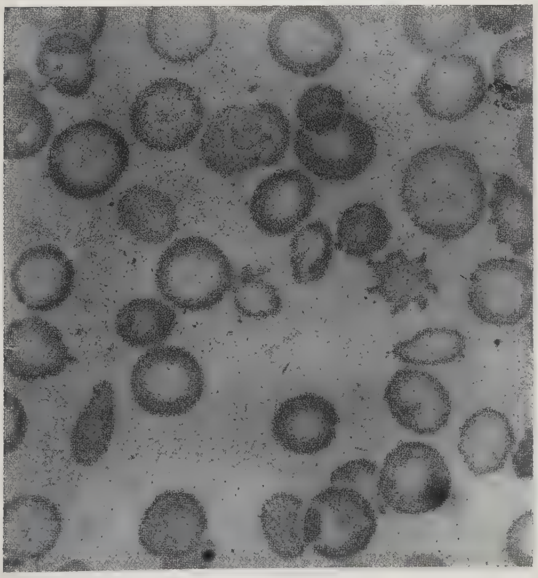


Fig. 425. Blood film of hypochromic anemia showing hypochromia, poikilocytosis, and anisocytosis. (×950.)

Burr cells: These are irregularly shaped erythrocytes and triangular cells which have been described in hemolytic episodes and in uremia.

Schistocytes: These are irregularly fragmented cells.

Heinz bodies: These are seen in preparations stained with supravital dyes and consist of denatured globin (for details, see previous discussion of Heinz bodies).

Reticulocytosis: In hemolytic anemias there is a marked increase in reticulocytes (see previous discussion of reticulocytes). The exception occurs in aplastic crises.

Macrocytosis: This accompanies increased erythropoiesis and may be the result of release of large numbers of reticulocytes.

Siderocytosis: See previous discussion of siderocytes.

Bone marrow findings: There is marked erythroid hyperplasia involving all red cell precursor forms. It is characteristic that the red cell precursors fail to show the morphologic changes which affect the mature red cells and are responsible for the hemolytic process. The myeloid-erythroid ratio is altered so that it may become 1:1 or even reversed, 1:2 or 1:3. Compared with the normal erythropoieses, there is usually a more pronounced increase of proerythroblasts and of erythroblasts so that they are about equal in number to the normoblasts. The hyperplastic erythroid marrow replaces the fatty marrow in the long bones and may expand the flat, short tubular bones. The marrow cavities of these bones become too small to accommodate the growing marrow so that the bony trabeculae as well as the cortices thin out, producing characteristic changes in the x-ray pattern.

Myeloid-erythroid hyperplasia has been classified according to its effectiveness into effective and ineffective erythropoiesis (see discussion of ferrokinetics) as well as into the following 3 types—compensated, decompensated, and dyserythropoietic.

Compensated hyperplasia implies production of a sufficiently increased number of mature red cells which are able to counteract the lytic process so that no anemia develops. If the red cell destruction is of considerable magnitude, an anemia will develop but will not be progressive, representing a stable balance between formation and destruction of red blood cells.

Decompensated erythroid hyperplasia implies that the process falls short of counterbalancing the red blood cell destruction so that immature red blood cells are released into the circulation, such as marrow reticulocytes, nucleated red cells, etc., and a progressive anemia develops.

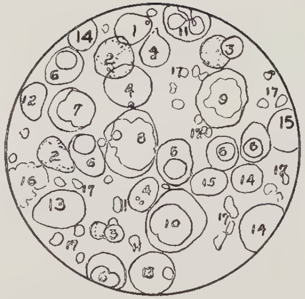
The dyserythropoietic marrow is quite unable to respond to the lytic process by the required degree of hyperplasia so that a severe progressive anemia develops without any evidence of marrow stimulation and hyperplasia, such as nucleated red cells, marrow reticulocytes, etc.

The bone marrow erythroid hyperplasia is reflected in the peripheral blood by reticulocytosis, macrocytosis, and the appearance of young nucleated forms (erythroblastemia).

Crises

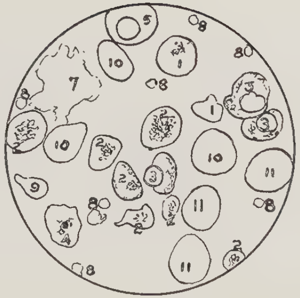
In all hemolytic anemias there occur periods of acute exacerbation which are called

Idiopathic hemolytic anemia



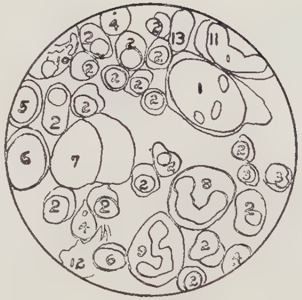
Blood film
(Giemsa stain; ×950)

1. Marginal granules
2. Basophilic punctation
3. Normoblast with basophilic punctation
4. Howell-Jolly bodies
5. Normoblast with Howell-Jolly body
6. Normoblast
7. Lymphocyte
8. Monocyte
9. Basophilic myelocyte
10. Neutrophilic myelocyte
11. Segmented neutrophil
12. Erythrocyte showing karyogenic metachromasia
13. Endoglobular degeneration
14. Target cells (?)
15. Polychromatic erythrocyte
16. Fragmentary nuclear structure
17. Blood platelets; erythrocytes showing anisocytosis, poikilocytosis, polychromasia, basophilic punctation, and some hypochromia



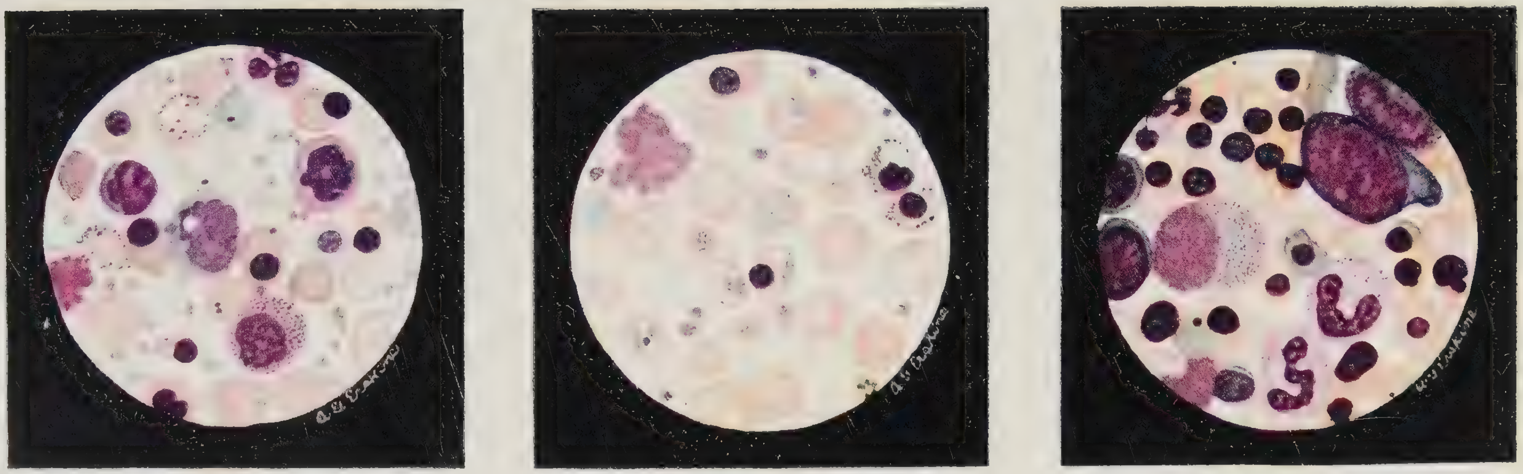
Blood film—reticulocytes
(supravital stain; ×950)

1. Marginal granule in reticulocyte
2. Reticulocyte
3. Normoblasts with stained reticulum
4. Reticulocyte with Howell-Jolly body
5. Normoblast
6. Howell-Jolly body
7. Fragmentary nuclear structure
8. Blood platelet
9. Poikilocyte
10. Target cells (?)
11. Hypochromic cells; marked anisocytosis and hypochromia



Bone marrow
(Wright-Giemsa stain; ×950)

1. Erythrogonia
2. Normoblast
3. Normoblast in karyorrhexis
4. Howell-Jolly body
5. Polychromatic macroblast
6. Lymphocyte
7. Promyelocyte
8. Neutrophilic juvenile
9. Neutrophilic stab cell
10. Segmented neutrophil
11. Eosinophilic juvenile
12. Fragmentary nuclear structure
13. Polychromatic erythrocyte



Idiopathic hemolytic anemia

Blood film

Blood film—reticulocytes

Bone marrow

Fig. 426

crises. They are of 2 types—hemolytic and aplastic.

In the hemolytic crisis there occurs a sudden increase in the hemolytic process often associated with further reduction of red cell life span. This crisis is frequently precipitated by an infection.[477]

In the aplastic or aregenerative crisis, in spite of continuing hemolysis, the bone marrow suddenly, but only temporarily, ceases to produce mature erythroid elements. The cause for this failure is unknown, but clinically it is often related to infections or immune mechanisms very similar to the hemolytic crisis. The clinical expression is a rapid precipitous aggravation of the anemia which lacks the usual reticulocytosis and marrow erythroid hyperplasia. The nonerythroid marrow elements are not affected.

In sickle cell anemia a third form of crisis has been described—thrombotic crisis.

Erythrokinetics: As the result of the hemolysis the serum iron is elevated, and this elevation is even more marked in hemolytic diseases in which there is interference with iron utilization. During the recovery period when the hemolytic process declines, the continuing marrow erythroid hyperplasia will produce a fall in the serum iron. Radioiron studies show rapid uptake of serum iron by the hyperplastic bone marrow and increased hemoglobin synthesis. Hemolysis can be increased to about 6 times the normal rate before iron deficiency occurs. The spleen shows increased radioactivity (iron) since most of the hemolysis takes place in this organ. The red cell survival time is, of course, shortened.

Radioiron studies have confirmed the impression that the spleen is always involved in hemolytic disease. Clinically it is usually enlarged. The destruction of red cells in the spleen may be due to several processes. Slowing of the blood stream may lead to sludging, autoagglutination of the red cells, or both. This process leads to metabolic changes in the red cells and enables the spleen to destroy them. That spherocytosis can be considered to be a prehemolytic state has been mentioned. It represents the result of a damaged cell membrane, and these cells, similar to sickle cells and other abnormally-shaped cells, are more easily destroyed. Last, the macrophages, members of the reticuloendothelial system, may engulf damaged or aged erythrocytes (erythrophagocytosis).

Osmotic fragility: Osmotic fragility is increased (see previous discussion).

Autohemolysis: Incubation of sterile defibrinated or oxalated blood at 37° C. shows only minimal spontaneous hemolysis during the first 24 or 48 hr., followed rather rapidly by autohemolysis. In hemolytic anemias the rate of spontaneous lysis is accelerated. This acceleration is due to several factors, among which spherocytosis, action of antibodies, and action of chemicals can be mentioned. The rate of hemolysis can be slowed by the addition of glucose, mannose, and certain nucleosides.

Mechanical fragility: Mechanical fragility is increased (see previous discussion).

HEMOLYTIC ANEMIAS DUE TO INTRACORPUSCULAR DEFECTS

Hereditary spherocytosis (congenital hemolytic jaundice; acholuric jaundice)

This is a hereditary disease transmitted as a mendelian dominant. In its clinical expression it extends from an almost normal carrier stage to a severe hemolytic anemia and from a disease appearing in early infancy and childhood to a condition which may manifest itself for the first time in the later years of life. The homozygous form may not be compatible with life, producing fetal death. Its chronic course is accentuated by more acute episodes and attacks of crises of varying frequency.

The outstanding clinical findings are anemia, jaundice, splenomegaly, and skeletal changes. The hemolytic findings are those of a hemolytic anemia with prominent spherocytosis, anisocytosis, some macrocytosis, and pronounced reticulocytosis.

Hematologic findings

Red blood cells are characterized by a shortened survival time and increased osmotic and autohemolytic fragility, especially after incubation. Whereas most hemolytic anemias show an increased fragility in hypotonic saline solution, it appears that congenital spherocytosis alone shows a decreased resistance to lysolecithin. This is an enzyme normally present in serum which, after having been adsorbed onto the red cells, leads to their destruction in the spleen. Cross survival studies show the previously discussed pattern of an intrinsic defect, the cells of hereditary spherocytosis disappearing rapidly in a normal recipient.

Pathophysiology of red blood cells

Splenectomy is clinically a curative procedure in patients suffering from hereditary spherocytosis, but it does not affect the red blood cell abnormality. The exact nature of the intrinsic defect is not known. Loehr described several enzymatic defects which produce abnormalities of the intracellular glycolytic metabolism. Such a defect can be proved by the incubation of red cells of hereditary spherocytosis with P^{32}. The P^{32} absorption is normal, but its utilization is abnormal. More P^{32} is used for the formation of inorganic phosphates and much less is incorporated into 2-3-diglycerophosphate and adenosine triphosphate. This defect can be corrected by the addition of glucose and nucleosides. It has also been shown that the cells of hereditary spherocytosis contain a slightly diminished amount of potassium.[478] It is possible that the metabolic defects described are related to changes in the red cell membrane which has been reported to contain a lessened amount of lipid. Not all cases of hereditary spherocytosis may have the same type of intrinsic defect. Allison[479] showed that the red cells of hereditary spherocytosis show a defect in the synthesis of phosphatidyl ethanolamine so that lysogenic compounds (lysophosphatidyl ethanolamine) accumulate within the red cells.

Anemia: The hemoglobin levels vary considerably from case to case and sink rapidly during a crisis. The peripheral blood film shows anisocytosis and Cabot rings and may contain siderocytes and Howell-Jolly bodies. The latter two are especially frequent after splenectomy. Supravital stains show numerous reticulocytes, many of which may contain only one single blue dot.

Leukocytes: The white blood cell count is not pathologic. During a hemolytic crisis it may increase and show a shift to the left, whereas in an aplastic crisis it may decline to leukopenic levels.

Bone marrow: The picture is that of erythroid hyperplasia and reticulocytosis. The latter, according to Rohr,[480] is characteristically less in the bone marrow than in the peripheral blood in cases of hereditary spherocytosis.

Plasma: Increase in nonconjugated indirect-reacting bilirubin, hence jaundice.

Nonhematologic findings

Urine: Increase in urobilinogen and urobilin.

Liver: Pigment stones in the gallbladder.

Spleen: Splenomegaly.

Skeletal system: Tower-head formation is often associated with deformities of the fingers and vertebrae. Bone marrow hyperplasia leads to widening of bones and to abnormal spacing of teeth and radiologic appearance of rarefaction. There is a sunray effect on x-ray films of the skull.

Eyes: Mongolian fold.

Treatment

Splenectomy (accessory spleens?) is the treatment of choice. The basic defect is not influenced, but the site of hemolysis is removed.

Enzyme-deficient hemolytic anemias

In this classification fall the hemolytic anemias produced by the action of certain drugs (primaquine, sulfanilamide, acetanilid, phenacitin, and other aniline derivatives, naphthalene, phenylhydrazine, Furadantin) and by certain legumes (fava bean and peas) in certain susceptible individuals. In most patients these substances do not produce a hemolytic anemia, but in a few they do. Such sensitivity was first noticed in American Negroes treated with primaquine for malaria. The common denominator of all these anemias is a congenital defect in the aerobic glycolytic pathway, a deficiency in glucose 6-phosphate dehydrogenase (G6-PD) activity. This defect is inherited as a sex-linked gene of intermediate dominance. Loehr has shown that this deficiency leads to a fall in the reduced glutathione, in triphosphopyridine nucleotide (TPN), and in diphosphopyridine nucleotide (DPN). These enzyme-deficient cells have a normal survival time, and hemolysis occurs only if they come in contact with one of the substances just mentioned. These substances apparently interact with oxyhemoglobin to form hydrogen peroxide (H_2O_2), which in normal cells is destroyed in the presence of reduced glutathione and glutathione peroxidase. In normal cells reduced glutathione is rapidly regenerated from the oxidized glutathione. In the face of G6-PD deficiency TPNH formation is inadequate, and glutathione is inadequately reduced so that H_2O_2 is not decomposed, and hemoglobin is subjected to oxidation and decomposition with the result of Heinz body formation and hemolysis.

The destruction mainly involves older cells which normally have a reduced glutathione, adenosine triphosphate (ATP), and DPN complement so that even in the face of continuing administration of any of the aforementioned toxic substances, the hemolysis ceases for a period of several weeks until the young cells, which enter the circulation with a normal complement of reduced glutathione, reach about the sixtieth day of their lives.

The hemolysis produced by the fava bean is not adequately explained as yet, though it may be related to the action of an anthraquinone derivative.

The hemolysis, which apparently is due to an intrinsic corpuscular defect, is intravascular in type and leads to hemoglobinuria but usually not to other manifestations of intravascular hemolysis.

Laboratory diagnosis of primaquine type of hemolytic anemia

The diagnosis of the primaquine type of hemolytic anemia and of the sensitivity of patients to these drugs prior to their administration can be established by several tests.

Beutler[481] found that in vitro drug-sensitive erythrocytes form small scattered Heinz bodies more readily and in greater number than do nonsensitive erythrocytes when they are incubated with acetylphenylhydrazine. The nonsensitive erythrocytes form only a few marginal somewhat larger Heinz bodies. Later Beutler[482] described a less subjective test based on the fact that in sensitive red cells the reduced glutathione level is lower than in nonsensitive cells. The **reduced glutathione stability test** consists of incubating blood samples with acetylphenylhydrazine and measuring the reduced glutathione level before and after incubation.

The latest method of investigation is the determination of glucose 6-phosphate dehydrogenase level. This level is measured indirectly by measuring the rate of TPNH formation. This reaction is based on the following reaction which is catalyzed by glucose 6-phosphate dehydrogenase.

Glucose 6-phosphate + TPN → 6-Phosphogluconate + TPNH

It was also found that as TPNH is formed it reduces brilliant cresyl blue dye to a colorless form. The rate at which the color of the dye disappears can be followed visually and is proportional to the glucose 6-dehydrogenase content of the red cells. A colorimeter is not required. This procedure appears to lend itself to mass screening. The dye has been stabilized and a simple test perfected[483] wherein 0.3M TRIS buffer and a red cell hemolysate are added to a vial containing the dye and a stabilizer.

Pathophysiology

Danon[484] examined the glucose 6-phosphate dehydrogenase-deficient erythrocyte with the electron microscope and found that the enzyme-deficient cells have a smooth cell membrane, whereas control cells show a granular pattern. In normal peripheral blood the granular pattern is characteristic of young cells and is present in 70% or more of all cells. The smooth pattern, on the other hand, in normal blood is characteristic of old cells. Electron microscopic examination of drug-sensitive blood shows a reversal of the normal ratio so that smooth cells predominate (60% or more). This morphologic change alone cannot be the etiologic factor of enzyme-deficient hemolytic anemias, as these cells, when not exposed to drugs, etc., have a normal survival time.[485, 486]

The drug-sensitive cells lack an activator of G6-PD which is present in the stroma of normal cells.

Waller[487] showed that the erythrocytes of some patients are almost devoid of glucose 6-phosphate dehydrogenase, whereas Dacie[488] was able to show complete arrest of glycolysis in other cases.

Congenital Heinz body anemia

Gasser[489] described congenital Heinz body anemia in premature infants who developed hemolytic jaundice in the first few days of life. Later Veradi[490] found a similar disease in full-term children. The association of Heinz body and methemoglobin formation has been discussed previously. As methemoglobinemia frequently follows drinking of nitrite-rich well water, it has been suggested that intestinal bacterial nitrite formation may be related to this type of anemia. Normal erythrocytes are rich in the enzyme catalase which splits H_2O_2 into oxygen and water, whereas the erythrocytes of infants contain a diminished amount of this enzyme. There is experimental evidence available that lowered catalase activity is associated with increased tendency to Heinz body formation. This concept of a congenital absence or diminution of this enzyme is supported by the findings in those enzymatic deficient anemias in which Heinz bodies are seen when the anemia follows the ingestion of aniline dyes, sulfonamide drugs, naphthalene, etc. There is also experimental evidence which links hyposplenism (splenic agenesis and splenectomy) to Heinz body formation.

Hereditary (congenital) nonspherocytic hemolytic anemia[491]

This classification includes a number of chronic hemolytic anemias which are due to an intrinsic enzymatic corpuscular defect. They vary in expression of the hemolytic process, showing variations in red cell morphology, osmotic fragility, and clinical course. They are united by such features as absence of spherocytosis and thus fail to show increased fragility, and though they are congenital, a good family history is often lacking. They are most commonly found in subjects of British and Northern European descent. Splenectomy is not followed by clinical improvement.

Selvyn and Dacie[492] differentiate 2 types which cannot be distinguished clinically but which show changes in autohemolytic behavior.

Type I
Normal autohemolysis of whole blood decreased by the addition of glucose but not corrected up to the normal response

Type II

Increased autohemolysis of whole blood not corrected by the addition of glucose

Hematologic features

The morphologic picture varies considerably from case to case, but slight anisocytosis is evidenced by the presence of macrocytes, normocytes, occasional elliptocytes, and target cells. Slight reticulocytosis and polychromasia may be seen. The osmotic fragility before incubation is normal in both types but is increased in type II after incubation.

Other types of congenital hemolytic anemia

Such anemias have been described in congenital porphyria[493] (erythropoietic porphyria) in which the cells fluoresce under ultraviolet light since they contain an increased amount of free erythrocyte protoporphyrin (fluorescytes).

Normal erythrocytes contain a small amount of free porphyrin (erythrocyte protoporphyrin [E.P.]).[494, 495] In cases of impaired hemoglobinization, there occurs an increase in E.P., indicating inadequate utilization of protoporphyrins in the process of hemoglobin synthesis. An increase of E.P. is seen in iron-deficiency anemia and in regenerative hemolytic anemias; normal values are found in pyridoxine deficiency and in pernicious anemia (see discussion of porphyrins).

Thalassemia

Thalassemia is a hemolytic anemia associated with an inherited intracorpuscular defect of erythrocytes. First differentiated by Cooley and Lee,[496] it has often been referred to as Cooley's anemia. This syndrome has also been designated Mediterranean anemia or Mediterranean disease. The latter designation has been applied chiefly because the syndrome has occurred most frequently in people of Mediterranean stock, with Italians predominating. Although scattered cases had been described in non-Mediterranean stock, it was not until Minnich and co-workers[497] reported 32 cases in Thailand that any considerable number had been collected outside of this group. In subsequent studies, most of these cases were found to be a combination of Mediterranean-E hemoglobin disease rather than pure thalassemia.[498] It is quite generally agreed today that the syndrome is due to an inherited characteristic of the erythrocyte as a mendelian dominant. Two major subdivisons are recognized: (1) the symptomatic disease form, thalassemia major, and (2) an asymptomatic form, thalassemia minor. Thalassemia major is the homozygous disease form with a double dose of the gene for the syndrome, whereas thalassemia minor is the heterozygous trait form, having only one gene for Mediterranean disease. In addition to these major divisions, studies of electrophoresis and alkali denaturation have revealed combinations of thalassemia and molecular disease of hemoglobin. Examples of such combinations are sickle cell-thalassemia disease, thalassemia-C disease, and thalassemia-E disease (see discussion of molecular disease of hemoglobin, pp. 1280ff). The combination of sickle cell-thalassemia is sometimes referred to as microdepranocytosis.

Other combinations are with hemoglobin-G, hemoglobin-H, and Bart's hemoglobin. When hemoglobin-E is present, there is usually no other hemoglobin detectable by electrophoresis. The presence of hemoglobin-H is revealed by intraerythrocytic inclusions which can be seen with supravital stain. It is possible that the thalassemia gene potentiates the abnormal hemoglobin genes. Abnormal hemoglobins are inherited by a single gene.

Thalassemia major

In the disease form anemia is a striking feature, varying in intensity from patient to patient, but often of sufficient severity to cause symptoms. An outstanding feature of the disease is the x-ray appearance of the skull which shows perpendicular striations suggesting hair standing on end. Involvement of other bony structures may be present, and in the hands it takes on a mosaic pattern on x-ray examination. These changes are the result of bone marrow hyperplasia. They also lead to characteristic facial expressions and changes such as a round skull, a broad nose with an indented bridge, and a mongolian type of inner canthal folds.

Splenomegaly may occur and at times reaches fairly high proportions. The hemolytic crises are often attended by febrile periods, but jaundice is infrequent. As a result of the anemia, debility, and perhaps a lessened resistance, intercurrent infections are fairly frequent, and life expectancy is considerably reduced so that many of these patients do not live beyond childhood.

Hematologic findings

The blood picture in the disease form shows an anemia of variable degree, ranging from 1,000,000 up to 3,500,000 red cells/c.mm.

There is a pronounced lowering of the hemoglobin and hematocrit values, with a resultant hypochromic microcytic anemia. The blood spread not only reflects this hypochromasia and microcytosis but also shows marked poikilocytosis and anisocytosis. During and shortly following a hemolytic crisis, nucleated erythrocytes are commonly seen in the blood spreads, and Howell-Jolly bodies may be present. Target cells in varying numbers are so regularly encountered that their presence in a blood spread should always suggest thalassemia as a possible diagnosis. These cases are more common in hemoglobin-C disease. The target cells (leptocytes or platycytes) are hypochromatic and thin. They may break easily and may give rise to schistocytes. The end result of all these morphologic changes is a picture of markedly deformed red cells frequently represented by hemoglobin rings only. The red cell diameter may vary from 2-10μ. Electron microscopic pictures of the red cells show iron granules or ferritin and abnormal hemoglobin derivatives deposited within the cytoplasm. These changes are not seen in thalassemia minor and indicate poor iron utilization of thalassemic red cells.

Reticulocytes are often increased. There are no significant changes in the platelets. The leukocyte count may be considerably elevated during a period of hemolytic crisis. The bone marrow is usually hyperplastic, displaying a normoblastic hyperplasia which is not a specific characteristic enabling a diagnosis of the disease. Nests of foamy reticulum cells can be found at times.

Fragility studies of the erythrocytes show either a normal osmotic fragility or may show an increased resistance in hypotonic saline. This increase is partially explained by the flattening and thinning of the red blood cells in thalassemia. It is an important differential point in cases in which the blood picture because of its anisocytosis resembles hereditary spherocytosis. On the other hand, the mechanical fragility is increased.

Red cell survival studies reveal a diminished survival time in some cases and a normal survival in others.[499] The observations of Singer et al.[500] that thalassemia major displays a considerable increase in hemoglobin-F have been verified by many observers.

The hemoglobin in thalassemia is thus a mixture of hemoglobin-A and hemoglobin-F. The F component is similar to fetal hemoglobin and may vary in amount from 10-80% or more. The amount is not related to the severity of the disease, but the presence of hemoglobin-F is one of the characteristics of all thalassemias. With starch block electrophoresis another hemoglobin can be identified which is normally not present at birth. It is seen in low concentration in other anemias, but is present in high concentrations in thalassemia minor. This hemoglobin is labeled hemoglobin-A_2. In lower concentrations it is also found in thalassemia major. At the age of 3 mo., up to 3% may be found in peripheral blood; in various anemias the level may rise to 4%; in thalassemia it may vary from 1-9%, whereas in thalassemia minor it rises to 13%. There is no relationship between hemoglobin-A_2 and hemoglobin-F except that when hemoglobin-F concentrations are high hemoglobin-A_2 concentration is usually low. The finding of hemoglobin-A_2 is valuable in the diagnosis of the carrier state (heterozygous form).

Nonhematologic findings

Serum findings: Nonconjugated bilirubin is increased. Serum iron is elevated, and iron binding capacity is low. The latter feature is an important point in differentiating thalassemia from severe iron-deficiency anemia.

Urinary findings: Urobilinogen is increased; amino acid uria has been described. The latter may be an expression of the hemolytic process or may be due to kidney or liver damage.

Thalassemia minor

In thalassemia minor all signs and symptoms of thalassemia major may be present but to a lesser and markedly variable degree. Despite apparently similar genetic background (heterozygosity for the thalassemia gene), these patients show extreme variations in symptoms so that etiologically gene interaction or protective mechanisms must be considered. The fetal hemoglobin may vary from 2-12% or may be absent. As pointed out previously, in most heterozygous patients hemoglobin-A_2 is increased diagnostically.

Thalassemia intermedia

Thalassemia intermedia[501, 502] is a clinical term denoting clinical and hematologic features ranging between those observed in thalassemia minor and thalassemia major. These patients usually include young adults or adults who more or less show the features of thalassemia major. They differ from thalassemia major by the very fact that the homozygous form of Cooley's anemia usually leads to death in early childhood. Genetic studies were made in some of the reported cases, and it could be shown that both parents had thalassemia minor. The children therefore had to be considered to be suffering from the homozygous form of Cooley's anemia. The electrophoretic hemoglobin pattern shows the presence of fetal hemoglobin and hemoglobin-A_2.

Pathophysiology of thalassemia

As previously mentioned, Heilmeyer classifies thalassemia in the sideroachrestic anemias since the thalassemia gene interferes with the heme synthesis. There is also evidence that thalassemia is the result of a biochemical change in the hemoglobin molecule which may be the result of mutations which affect the alpha or beta hemoglobin polypeptides. The thalassemia gene apparently suppresses beta polypeptide production so that gamma polypeptide production (fetal hemoglobin) is free to assert itself (see discussion of abnormal hemoglobin).

Last, hemoglobin metabolism[503] studies suggest not only the defect in hemoglobin synthesis but also an abnormality of erythrocyte production.

In the combinations of thalassemia and sickle cell hemoglobin-C or hemoglobin-E, the clinical and blood pictures appear to have a position intermediate between thalassemia disease and thalassemia trait. Although sickling is not present in thalassemia alone, sickling of the red cells is demonstrable when it combines with the sickle form. Electrophoretic studies in these combinations demonstrate the abnormal hemoglobin-S, hemoglobin-C, or hemoglobin-E, as well as hemoglobin-F. However, attention is again called to the fact that hemoglobin-F has a mobility approximating that of hemoglobin-A and cannot be readily demonstrated by paper electrophoresis.

Paroxysmal nocturnal hemoglobinuria

Paroxysmal nocturnal hemoglobinuria,[508, 509] often referred to as the Marchiafava-Micheli syndrome, is associated with a chronic hemolytic anemia with acute paroxysms of hemolytic crises occurring at night. These crises are of sufficient degree to result in hemoglobinuria. Although the red cell is the predominant hemopoietic element involved, the white cells and platelets may also be affected. Crosby[504] has presented a magnificent review of this syndrome, together with many of his original observations. He concludes that paroxysmal nocturnal hemoglobinuria is an acquired disease of the hemopoietic system in which abnormal red cells, white cells, and platelets are then susceptible to some normal plasma factor which is believed to be properdin.[505] This serum euglobulin is distinct from complement and the components of the clotting system but requires complement and magnesium ion for its activity. Crosby calls attention to the fact that hemolytic crises occur when the patient is asleep (whether at night or day), and that during this period changes in the balance of an inhibitor-hemolysin system may account for the crises. Other investigators have felt that changes of pH are produced by retention of CO_2 during sleep; this observation forms the basis of the Ham[506] acidification test and its modifications.

In addition to the anemia, these patients develop leukopenia which may become chronic and perhaps contributes to the increased susceptibility to infection that these patients show.

Thrombocytopenia is generally not marked, and purpura is uncommon. However, agglutination of platelets in this disease accounts for the vascular thromboses seen in paroxysmal nocturnal hemoglobinuria. There appears to be some evidence of a vicious cycle of competition between members of the coagulation mechanism (especially thrombin) and the factors involved in paroxysmal nocturnal hemoglobinuria. Dicumarol has had some beneficial effect in protecting these patients against thrombotic episodes and perhaps the development of anemia. Heparin, on the other hand, causes increased hemolysis.

We are thus confronted with a rather rare disease characterized by pancytopenia caused by at least 2 factors: (1) the plasma factor and (2) an intraerythrocytic factor.

Plasma factor

There is some doubt whether the complement-properdin-magnesium system is involved (or is the only plasma system involved), as Crosby[507] found that a thrombin-plasma factor system was linked to the hemolytic process, the plasma factors being in the nature of antibodies.

Intraerythrocytic factor

Paroxysmal nocturnal hemoglobinuria is essentially due to an intraerythrocytic defect. Crosby[507] proved the existence of an abnormal ratio of erythrocytic fatty acids as well as an associated stromal defect. The latter can also be seen by electronmicroscopy but has been claimed to be artifactual by some observers. Acetylcholinesterase activity is subnormal, but a similar defect is exhibited by other diseases which do not have a hemolytic component. The cause of paroxysmal nocturnal hemoglobinuria is still not known. During an attack, both the mature peripheral red cells and the immature marrow erythroblastic cells hemolyze. The marrow responds only poorly and inadequately to the hemolytic red cell destruction.

Laboratory tests

Urine: Dark, especially notable after sleep.

Plasma: Usually darker in the morning than in the afternoon and evening.

Autohemolysis: Rapid at 37° C.

Ham test (acid serum test):

1. Acidify 0.5 ml. serum by adding 0.05 ml. 2.2N HCl acid.
2. Control—0.5 ml. unacidified serum.
3. Add 1 drop 50% suspension of patient's washed cells to each serum, incubate at 37° C. for 1 hr. and then centrifuge.

If the cells are from a case of paroxysmal nocturnal hemoglobinuria, marked hemolysis occurs after 15 min.

Thrombin test (Crosby):

1. Prepare an acidified red cell and serum mixture and control as for the acid serum test.
2. To 1 ml. each mixture add 100 units bovine thrombin dissolved in 0.1 ml. saline.
3. Incubate both tubes at 37° C. for 15 min.

If the cells are from a case of paroxysmal nocturnal hemoglobinuria, more hemolysis occurs in the presence of thrombin.

Blood picture: This has been described previously. Anemia and reticulocytosis are only slight.

Paroxysmal cold hemoglobinuria

This condition is found in syphilis and is often acutely brought on by sudden chilling of the body surface. The urine is diagnostic; if taken directly after or during the attack, color varies from brown red to black; acid reaction; specific gravity depends on kidney function and water balance; on boiling, a brown coagulum of albumin develops; hyaline casts are present; spectroscopically, bands of hemoglobin and methemoglobin; there are no red cells in the urine; urine contains urobilin.

During the attack the following **Donath-Landsteiner autohemolysis phenomenon** may be found:

1. Place some blood in a test tube.
2. Chill to 5° C. for 10 min. Hemolysis will occur.

Normal blood will show no such hemolysis.

This test is based upon the fact that in this disease a special hemolytic substance has been liberated into the blood stream. In the blood picture there are leukopenia, hypochromia, and poikilocytosis during the attack.

Familial hemolytic anemia with erythrocyte inclusion bodies and defect in pigment metabolism

Schmid et al.[510] and Scott et al.[511] in 1959 and 1960 described several cases of a severe congenital hemolytic anemia characterized by Heinz body-like intraerythrocytic inclusions. These were also found in reticulocytes and in a few normoblasts. The erythrocytes contained siderocytic granules, basophilic stippling, and rodlike structures. In all reported cases splenectomy had been performed. The urine contained a dark brown pigment believed to be mesobilifuscin. Scott's patient also showed evidence of an abnormal hemoglobin, bringing to mind the hemolytic anemia occasionally associated with hemoglobin-H.

Molecular diseases of hemoglobin

Pauling's[512] discovery of the differences in electrophoretic mobility of normal and sickle cell hemoglobin has opened a rapidly growing field in the investigation of hemoglobin. The basic concept of this work is that hemoglobin exists in various molecular structures that differ from that of normal hemoglobin. These differences can be elucidated by electrophoresis, as well as by other methods such as gel formation,[513] tactoid formation in phase microscopy,[513-515] and alkali denaturation.[516] The technic of paper electrophoresis for studying proteins, including hemoglobin,[517, 518] has supplemented the much more complicated and expensive Tiselius apparatus and has opened the way to large-scale investigation of diseases associated with hemoglobin molecular disease.

The **nomenclature**[519] **of the various types of hemoglobin** now described on the basis of electrophoretic patterns are as follows: (1) Normal adult hemoglobin or hemoglobin-*A*, (2) normal fetal hemoglobin or hemoglobin-*F*,[516] (3) sickle cell hemoglobin or hemoglobin-*S*,[520] (4) hemoglobin-*C*,[520-522] (5) hemoglobin-*D*,[520, 523] and (6) hemoglobin-*E*.[524]

Hemoglobin-E was identified in 1954. Since then the following hemoglobins have been described: *G, H, I, M, J, K, N, O, L, P,* and *Q*. There are still others as yet not designated by letters, such as Lepore hemoglobin, Bart's hemoglobin, and others.[525, 526]

With the exception of hemoglobin-M, all abnormal hemoglobins show normal oxyhemoglobin dissociation curves.

Table 250. *Distribution of various types of hemoglobin*

	Hemoglobin type					
	A	*S*	*C*	*D*	*E*	*F*
Normal newborn	15-50%	–	–	–	–	50-85%
Normal child up to age 2½ yr.	97-100%	–	–	–	–	Up to 3%
Normal adult	98.4-100%	–	–	–	–	Trace
Sickle cell trait	55-76%	24-45%	–	–	–	Trace
Sickle cell disease	–	75-100%	–	–	–	0-25%
Hemoglobin-C disease	–	–	100%	–	–	Trace
Hemoglobin-C trait	60-75%	–	25-40%	–	–	Trace
Sickle-C disease	–	33-50%	50-67%	–	–	Less than 2%
Thalassemia-C disease*	25%	–	75%	–	–	Trace
Hemoglobin-D trait	51-65%	–	–	35-49%	–	–
Sickle-D disease	–	?	–	?	–	?
Thalassemia minor	90-100%	–	–	–	–	0-10%
Thalassemia major	0-60%	–	–	–	–	40-100%
Sickle-thalassemia disease	18-33%	67-82%	–	–	–	1-17%
Hemoglobin-E trait†	++++	–	–	–	+	–
Hemoglobin-E disease†	–	–	–	–	94-98%	2-6%
Thalassemia-E disease†	–	–	–	–	60-80%	20-40%

*Singer, K., et al.: Blood **9**:1032, 1954; Zuelzer, W. W., and Kaplan, E.: Blood **9**:1047, 1954.
†Chernoff, A. I., et al.: Proc. Cent. Soc. Clin. Res. **27**:26, 1954.

These hemoglobin types are inherited. Neel[527, 528] has reported the inheritance pattern of the sickling phenomenon in 75 kindreds. He maintains that this phenomenon is "due to a gene which in single dose (heterozygous condition) produces only the sickle cell trait, and in double dose (homozygous condition) sickle cell disease."* Translated into terms of abnormal hemoglobins, patients with sickle cell trait are represented by an S-A gene pattern, whereas those with sickle cell disease have an S-S pattern. The same principle applies to other combinations of abnormal hemoglobins. In the trait, quantitative electrophoretic studies show small amounts of abnormal hemoglobin in combination with relatively large amounts of normal hemoglobin. On the other hand, the disease syndrome reveals almost 100% abnormal hemoglobin. Motulsky and co-workers[529] report the following typical distribution of hemoglobins in various syndromes observed by them or collected from the literature. Table 250 is a modification, based on observations made subsequent to their report.

The electrophoretic mobilities of the various hemoglobins result in the following order: closest to the starting point is B_2, then follow C, E, (A_2), O, S, (D), L, (P), G, Q, A_1, A_3, (M, F), K, J, N, H and (I). This order applies to paper electrophoresis with veronal buffer pH 8.6. Parentheses () indicate that the hemoglobins cannot be separated from each other by this particular method. Such separation may be achieved by modified electrophoresis technics.

The mobility of hemoglobin-F so closely approaches the speed of hemoglobin-A that these two components are generally inseparable in the paper electrophoresis technic, although Smith and Conley[530] claim such separation is possible. In the Tiselius apparatus, the F component can be readily identified. In most clinical studies, the alkali denaturation technic[516] has been the method of choice for demonstrating hemoglobin-F. Hemoglobin-D has the same mobility as hemoglobin-S and cannot be separated by electrophoresis. Differences in solubility[520] have been used as a means of distinguishing these two components.

The presence of these various abnormal hemoglobins may or may not result in symptoms. In general, patients with a homozygous pattern and high percentages of abnormal hemoglobin show marked symptoms. Hemolytic crises with resultant anemia are prominent features. Associated with these crises are severe abdominal pain, fever, headache, hematuria, pallor, tachycardia, and dyspnea. Abdominal pain may be so severe as to suggest a surgical condition of the abdomen, and patients in this category have been mistakenly operated upon as emergency cases. Jaundice, as a result of excessive red blood cell destruction, is quite common, and hematuria is found occasionally. Vascular thromboses may result in a multiplicity of symptoms with the brain a fairly common site for such episodes. On physical examination the degree of pallor is proportionate to the anemia. Jaundice may be

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*From Neel, J. V.: Blood **7**:467, 1952.

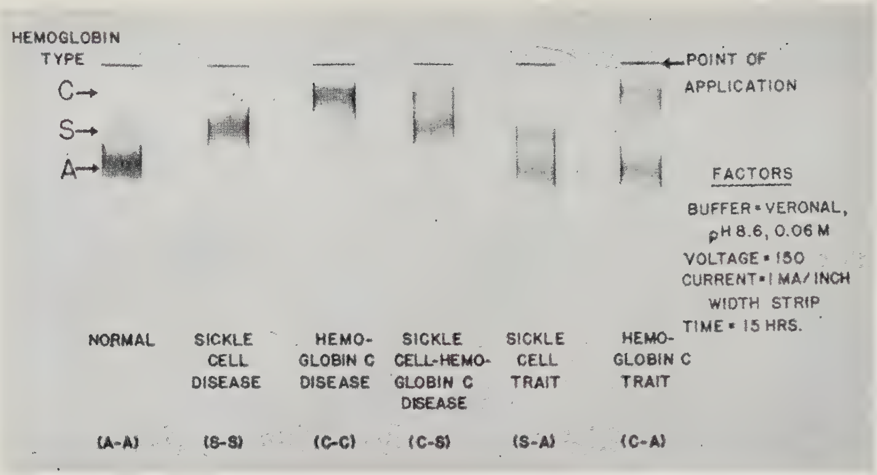


Fig. 427. Electrophoretic determination of hemoglobin types. Separation of hemoglobins by filter paper electrophoresis. (From Weinstein, I. M., Spurling, C. L., Klein, H., and Necheles, T. F.: Blood **9**:1157, 1954.)

present. Splenomegaly and sometimes hepatomegaly are quite common. In the heterozygous state, abnormal clinical findings are absent, and the patient's history is essentially negative. In combined states, such as sickle cell-hemoglobin-C disease, the clinical picture is generally intermediate in severity between the homozygous and heterozygous conditions. Although the picture is generally milder in such combinations, Smith and Conley[530] call attention to 2 patients in whom hemolytic crises were sufficiently severe to produce jaundice and profound anemia.

Just as the clinical pattern seems to follow the types of hemoglobin present, there also appears to be some parallelism in the hematologic studies. In the **homozygous** states, generally an anemia of varying degree occurs and may be quite profound. The stained blood spreads show numerous target cells, ranging from 60 to almost 100% of the red blood cells. During crises anisocytosis (chiefly microcytosis) and poikilocytosis are marked, and nucleated erythrocytes are common. Polychromasia is often seen. In those patients with sickle cell disease, sickled erythrocytes may rarely be seen in the blood spread. Osmotic fragility studies reveal an increased resistance. Mechanical fragility tends to be normal in an oxygen atmosphere but is increased in an atmosphere saturated with carbon dioxide. Reticulocytes are often increased, especially during or shortly after a hemolytic crisis. Siderocytes, which are iron-staining bodies within the erythrocytes, tend to be numerous.[531] Sickling is demonstrated only in patients with hemoglobin-S and may approximate 100% in some instances. In this respect, it is important to point out that sickling of such proportion occurs only in the homozygous S state. Homozygous C and E and thalassemia are not accompanied by sickling phenomenon. When these are combined with sickling such as sickle cell–hemoglobin-C disease, sickling will occur but to a lesser degree than in homozygous S.

Combinations of sickle cell and other abnormal hemoglobins, such as sickle cell–hemoglobin-C disease, show intermediate hematologic values. Kaplan, Zuelzer, and Neel[531] were particularly impressed with the markedly high target cell incidence in sickle cell–hemoglobin-C disease. Apparently variations in hematologic abnormalities may show the same wide variations that the clinical pictures display, ranging from relatively mild to very severe.

In the **heterozygous** or **trait** states hematologic studies reveal minimal deviations from normal. Anemia is not present. Target cells occur much more frequently than in normal people, but the percentage of such cells rarely exceeds 20%. Osmotic fragility tends to be normal or occasionally decreased, and mechanical fragility is generally normal or only slightly affected in CO_2. Siderocytes are absent or rare. Sickling in wet preparations is minimal, rarely exceeding 20% in subjects with hemoglobin-S.

From a clinical standpoint, the relationship of hemoglobin-F concentration to disease is not quite as clear-cut as with the other abnormal hemoglobins. Its high concentration in newborn infants (almost 100%) is not implicated in any disease process.

In the embryo a precursor form of hemoglobin-F may be present. In the fetus, hemoglobin-F may be carried by a red cell population different from the one carrying hemoglobin-A. In the first few months of extrauterine life, the percentage of fetal hemoglobin falls to about 10%; after 12 mo. it reaches the adult level of about 1%. It may be recapitulated that the presence of hemoglobin-F in the mother's circulation can be used as evidence of bleeding of the fetus into the maternal circulation (see discussion of hypochromic anemias).

Hemoglobin-F differs from adult hemoglobin in the following properties: (1) x-ray diffraction shows molecular differences between hemoglobin-A and hemoglobin-F, (2) fetal reduced hemoglobin is more soluble than adult reduced hemoglobin, (3) the amino acid composition of the globin moiety of fetal hemoglobin differs from the composition of adult hemoglobin, (4) the oxygen dissociation curve shows a higher affinity and loading capacity than adult hemoglobin and, (5) hemoglobin-F is easier oxidized than hemoglobin-A.

Singer and co-workers[516] originally demonstrated its association with sickle cell disease in which values ranging from 2-23.9% were found. Since this original publication, denaturation studies have taken on a pattern. Sickle cell–hemoglobin-C disease and sickle cell–thalassemia disease are both associated with high values for fetal hemoglobin. Thalassemia major is also associated with increased hemoglobin-F values. Mild elevation of hemoglobin-F has been noted in hemoglobin-E disease. When thalassemia is combined with other abnormal hemoglobins (e.g., thalassemia hemoglobin-E disease), fetal hemoglobin values are elevated. In the trait states of hemoglobin-S or thalassemia minor, the values are normal. Increased amounts of hemoglobin-F have been noted in some miscellaneous conditions, such as hereditary spherocytosis, chronic aregenerative anemia, untreated pernicious anemia, myelophthisic anemia, and some leukemia conditions often associated with extramedullary hematopoiesis.

Variations of hemoglobin-F have been described which are sensitive to alkali denaturation (Bart's hemoglobin and others).

Biochemistry of abnormal hemoglobins

In any given species the composition of the protein moiety of hemoglobin is genetically controlled so that no changes occur in its amino acid composition from generation to generation. On the other hand, if mutations occur (sometimes silent), the altered gene may produce changes in the amino acid sequence. The abnormal hemoglobins[531b] vary in their globin composition, whereas the heme component is the same for all hemoglobins. The abnormal hemoglobins have been designated by capital letters, as already stated, more or less in the order of their discovery. Two allelic genes control the synthesis of hemoglobin-A, hemoglobin-S, and hemoglobin-C, the latter 2 representing mutant forms of hemoglobin-A. Whether hemoglobin-F[531c] is also controlled by 2 allelic genes or is the result of environmental factors acting on a single cell capable of producing either hemoglobin-A or hemoglobin-F has not been decided as yet.

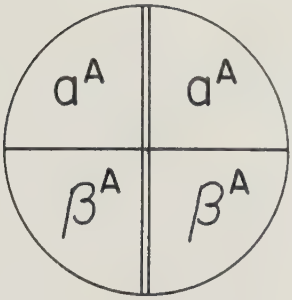


Fig. 428. Globin pattern of hemoglobin.

The globin moiety of hemoglobin can be visualized as a combination of 2 half-molecules, each half-molecule being a mirror image of the other. Each half-molecule contains 2 different polypeptide chains, an alpha chain and a beta chain, so that the entire molecule contains 2 alpha and 2 beta chains and can, in the case of hemoglobin-A, be represented by the formula $\text{alpha}_2{}^A/\text{beta}_2{}^A$.

Ingram[531a] by finger printing of hemoglobins found that amino acid changes in these polypeptide chains characterize each abnormal hemoglobin. He hydrolyzed or digested the protein into polypeptides and then separated them into individual peptides and amino acids by electrophoretic-chromatographic or chemical methods. Further electrophoresis in the presence of a detergent allowed allocation of the amino acids to one or the other of the chains. The composition of these polypeptide chains is controlled by 2 genes, one gene for each chain. These genes are not linked and are probably situated on separated loci. Any mutation in one or the other genes will result in changes in the amino acid composition of the chain.

Hemoglobin-A contains glutamic acid in

the beta chain, whereas in hemoglobin-S the same position is occupied by valine, and in hemoglobin-C it is occupied by lysine. Fetal hemoglobin contains a different arrangement of amino acids called the gamma chain. In each half-molecule it also contains an alpha chain as it occurs in hemoglobin-A. Hemoglobin-F is therefore represented by the formula $\text{alpha}_2{}^{A}$ $\text{gamma}_2{}^{F}$. Changes in the alpha chain have been found in hemoglobin-I. Hemoglobin-A_2 is characterized by a delta chain.

Investigation of abnormal hemoglobins

Identification and estimation of fetal hemoglobin—Chernoff-Singer

Alkali denaturation[534, 535]

Preparation of hemoglobin solution:

1. Collect approximately 5 ml. blood and add to a graduated tube containing anticoagulant; either heparin or ammonium potassium oxalate is satisfactory.
2. Wash the red cells 3 times with 0.85% sodium chloride solution.
3. Centrifuge for 10 min. at 2500 r.p.m. after the final wash and draw off the saline.
4. Measure the packed volume of cells.
5. Add 1.6 vol. distilled water to the packed cells, suspend the cells, and mix thoroughly.
6. Add approximately one fifth the total volume of toluene and shake vigorously for 5 min.
7. Centrifuge at 2500-3000 r.p.m. for 30 min.
8. Remove the toluene layer and any denatured protein which may appear at the water-toluene interface with a capillary pipet.
9. Filter the hemoglobin solution through a small retentive filter; if not clear, return the filtrate to the filter a second time.

This hemoglobin solution may be used in the procedure described in the following section.

Determination:

Reagents:

1. 0.0833N Potassium hydroxide solution.
2. Acid ammonium sulfate solution.
   (a) Make a saturated solution of ammonium sulfate by suspending 900 gm. ammonium sulfate in distilled water to a total volume of 1000 ml. and dissolve by heating.
   (b) Let the solution cool to room temperature and leave for at least 24 hr. for the excess ammonium sulfate to crystallize.
   (c) To prepare the reagent dilute 200 ml. this saturated solution with 200 ml. distilled water, add 1 ml. concentrated hydrochloric acid, and mix well.

Procedure:

1. Measure 3.2 ml. potassium hydroxide solution into a test tube.
2. Add 0.2 ml. hemoglobin solution, mix quickly, and let stand exactly 60 sec.
3. Add 6.8 ml. acid ammonium sulfate, mix well by inversion, and filter. If necessary, pour through the same filter again. This filtrate will contain any fetal hemoglobin which may be present.
4. Determine the OD solution at 540 mμ using distilled water as a reference.
5. Dilute 20 c.mm. original hemoglobin solution in 5 ml. distilled water.
6. Determine the OD this solution at 540 mμ.

Calculation:

$$\frac{\text{OD filtrate} \times 100}{\text{OD original Hb sol.} \times 5} = \%\ \text{Fetal Hb}$$

NOTE: Any spectrophotometer or any filter photometer having a filter that gives maximum transmission at about 540 mμ may be used. If the scale of the instrument is graduated in transmittance only, the % may be determined and converted to optical density using the following formula:

$$\text{OD} = 2 - \log \%\ \text{T}$$

Any cuvet having a diameter between 12 and 20 mm. may be used.

Recognition of fetal red blood cells in the maternal circulation

Normal adult blood may contain up to 2% fetal hemoglobin. Higher values have been reported in mothers of infants suffering from erythroblastosis fetalis or from nonhemolytic anemia due to bleeding into the maternal circulation. The recognition of fetal cells in the maternal circulation may be of some importance in the prevention of erythroblastosis. Since rapid destruction of fetal cells in the maternal circulation in A-B-O incompatibility apparently protects the mother from Rh sensitization, it is thought, that in the absence of A-B-O incompatibility, therapeutic destruction of fetal cells in the maternal circulation may prevent erythroblastosis fetalis. Such therapy will depend on the early recognition (post-delivery) of fetal cells in the mother's blood.

In 1957 Kleihauer[532] described a modification of the Wright-Giemsa stain employing McIlvaine phosphate buffer with a pH of 3.4 (14.3 ml. citric acid and 5.7 ml. disodium phosphate) which allows differentiation of normal hemoglobin-A containing erythrocytes from erythrocytes containing hemoglobin-F. His method was originally used as a rapid screening test for thalassemia and other congenital anemias and is based on the fact that normal erythrocytes are not acid resistant, whereas fetal cells are acid resistant.[533]

Procedure:

1. Air dry films of peripheral blood.
2. Fix in 80% ethyl alcohol.
3. Wash.
4. Immerse in McIlvaine buffer for 10-15 min.
5. Wash.
6. Stain with Wright-Giemsa.
7. Dry and examine with high dry objective.

Interpretation: Normal (hemoglobin-A) erythrocytes are represented by shadows only. Fetal (hemoglobin-F) erythrocytes are acid resistant and remain intact.

Apt test

This test is used to distinguish ingested maternal blood from fetal blood in cases of gastrointestinal hemorrhage of the newborn and is based on the alkali resistance of fetal hemoglobin.

Method:

1. Suspend a small amount of bloody meconium in 10 ml. distilled water and add 0.2 ml. 10% NaOH.

If the solution turns opaque brown, the blood is from the mother, and if it remains pink, the blood is of fetal origin.

Electrophoresis[536]

1. Use horizontal bridge and veronal buffer solution of pH 8.6, ionic strength 0.06.
2. Use horizontal bridge and power supply with 500-volt outlet and Whatman electrophoresis paper cut into strips according to the width and length of the horizontal bridge.
3. Fill the chambers at both ends of the bridge with buffer and wet the filter paper with buffer by immersing it completely.
4. Blot the electrophoresis paper between 2 pieces of filter paper and place the blotted electrophoresis paper over the horizontal bridge. Make sure that the ends of the paper are inserted into the buffer-containing chambers at either end of the apparatus.
5. Make a vertical pencil line at about the center of the paper.
6. Apply small drops of hemoglobin solution approximately 0.003-0.005 ml. at small marks on the pencil line.
7. Connect the electrodes to the power supply, adjust to 350 volts and 12-15 ma.
8. Allow the electrophoresis to continue for 10-12 hr. At the end of the run, dry strips immediately in oven.

The original method employs 2 siliconized glass plates, one of which supports the electrophoresis paper while the other one covers it. It is held in place by clamps which distribute the pressure evenly.

Other methods of investigation of abnormal hemoglobins

These methods include tests for sickling (described in the discussion of sickle cell anemia), crystal violet stains to emphasize target cells, which occur in hemoglobin-C disease, and examination of unstained blood for intraerythrocytic inclusions, which occur in hemoglobin-C and hemoglobin-H disease.

Sickle cell anemia

Sickle cell anemia was first seen by Herrick[537] who observed peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. He did not recognize this as a distinct clinical entity, but it is now recognized as such. The disease is a familial one occurring mainly in Negroes or mulattoes. It was generally believed that it was an intrinsic characteristic of the Negro until Cooley and Lee[538] reported a case in a 4-year-old American-born Greek boy. Since then, other cases have been reported in Italians, in Sicilians, and even in a boy of Scottish-Irish parentage. It has been found as a latent phenomenon in 6 or 7% of several series of supposedly normal Negroes. L. W. Diggs of the University of Tennessee has made a special study of this type of anemia. He states that the average age of death of these patients is 11 years.

In West Africa, the ancestral home of most Negroes, sicklemia received the attention of Russell and Taylor in 1932, Smith in 1934, Reid in 1936, and Evans in 1944 and 1945. Findlay, Robertson, and Zacharias[539] reported on the incidence of sickle cell anemia in the British West African colonies. In all, 5500 Africans were examined, of whom 12.4% showed the sickling trait. One half of the number were natives of the Gold Coast and French Togoland; the remainder came from Gambia, Sierra Leone, and Nigeria. Sickling was found in all tribes and no significant specific tribal differences were noted; 11.2% were males and 12.6% were females.

The protective mechanism of balanced polymorphism described in the discussion of hemoglobin-F applies also to hemoglobin-S. The protection may be due not only to the presence of hemoglobin-S but also to an increased level of gamma globulin found in the protected children.[540]

Clinical features of sickle cell disease (homozygous sickle cell anemia)

Sickle cell disease occurs in about 2 per 1000 American Negroes. There is high infant and child mortality with an early (several months of age) appearance of anemia. Enlargement of the liver and spleen, followed after many years by contraction of the spleen, is encountered. Frequent complications are leg ulcers, osteomyelitis, and heart disease. In addition to the 2 types of crises (hemolytic and aplastic)[541] occurring in all hemolytic anemias but rarely in sickle cell disease, there occur thrombotic crises in hemoglobin-S disease. These are due to vascular occlusions produced by the interlocked sickle-shaped cells.[542]

Sickle cell trait

Sickle cell trait is found in about 10% of American Negroes and is asymptomatic in most patients. Some patients show hematuria (renal congestion?) and may develop splenic infarcts or pulmonary infarcts during poorly pressurized high altitude flight and during anesthesia.

Blood picture

The blood picture in sickle cell anemia shows sickle-shaped erythrocytes, often not typical in stained spreads, but 75-100% are distorted in moist preparations. There is present a normocytic normochromic anemia with normal MCV and normal MCHC. Signs of regeneration in the blood are seen in an increase in reticulocytes, diffuse basophilia, the presence of nuclear particles, and nucleated red cells. There are anisocytosis, anisochromia, and poikilocytosis.

The differential blood count is variable, usually with a left shift of the leukocytes. There is also an increase in blood platelets. Bleeding and coagulation times are normal. There is a decrease in fragility, but normal sedimentation.

No hematologic changes are noted in sickle cell trait.

Demonstration of sickling of erythrocytes

Method I—in vivo anoxia of Scriver-Waugh[543]**:**

1. Place a rubber band around the finger for 5 min.
2. Obtain a drop of blood from the finger and quickly place it under a cover slip.
3. Seal the edges of the cover slip with petrolatum.
4. Observe for sickling for several hours.

Method II—metabisulphite method of Daland-Castle[544]**:**

1. Add 1 drop blood to 2 drops freshly prepared 2% aqueous solution sodium metabisulphite on a glass slide.
2. Cover with a cover slip and observe in 15-30 min.

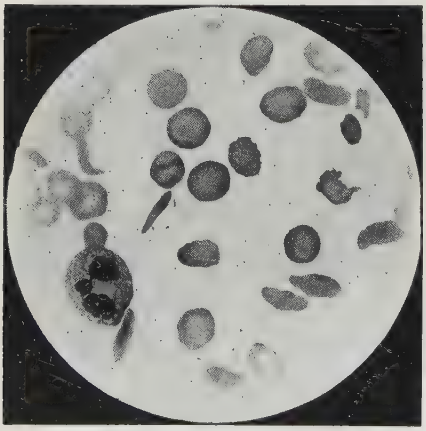


Fig. 429. Sickle cell anemia, stained film. (×950.)

With this method, sickling occurs promptly. In sickle cell **disease** 60-80% of the red cells may show sickling, whereas in sickle cell **trait** only a small percentage (5%) may show sickling.

To obtain permanent films, English[545] suggested that sickling be allowed to occur between 2 slides and these 2 slides then be separated in formaldehyde fumes released from heated formaldehyde tablets.

Electrophoresis of hemoglobin-S

In **sickle cell disease** the hemoglobin appears to be almost pure hemoglobin-S. A small amount of hemoglobin-F may be present. In infants, hemoglobin-F is seen naturally, but if it appears in older persons, the possibility of sickle cell–thalassemia disease must be considered. Hemoglobin-A is not present except when the pattern is obtained on a recently transfused patient.

In **sickle cell trait,** hemoglobin-S and hemoglobin-A can be identified. In sickle cell trait the hemoglobin-S component may amount to 45%, the remainder being hemoglobin-A. In comparison, in sickle cell disease, hemoglobin-S is usually over 50% and may reach 80-90%. Homozygous hemoglobin-S disease is represented by the following gene pattern; alpha-2^Abeta-2^S. The heterozygous form is represented by alpha-2^Abeta-2^A (hemoglobin-A) plus alpha-2^Abeta-2^S (hemoglobin-S). As stated previously hemoglobin-S is thought to be an allele of hemoglobin-A on the same locus.

Ferrohemoglobin solubility[545a]**:** The low solubility of reduced hemoglobin-S (ferrohemoglobin-S) may be used to differentiate hemoglobin-S from hemoglobin-D, which has identical electrophoretic mobility at pH 8.6.

Bone marrow findings

Bone marrow studies in sickle cell anemia have not been touched upon with much frequency. Sydenstricker[546] found the bone marrow to be abundant, bright red, and thin. Huck[547] and Sharp and Schleicher[548] confirmed these observations.

The bone marrow shows mainly erythroid hyperplasia involving all precursor forms of red blood cells. The marrow hyperplasia eventually produces bone changes which are similar to those seen in thalassemia, but in contrast to thalassemia radiologic evidence does not appear till adolescence or even later. The bone marrow may contain infarcted areas. In thalassemia infarcts are not usually seen. Rarely, megaloblastic erythropoiesis occurs in hemoglobin-S disease.

Nonhematologic findings

Urine: The presence of amino-aciduria has been reported. The common finding is a low specific gravity, as the kidneys in hemoglobin-S disease have lost some of their concentrating ability. An interesting common finding is the presence of hematuria in often unsuspected cases of sickle cell trait.

Plasma: There is a slight increase in gamma globulins and in the indirect van den Bergh test.

Pathology

Sickled red blood cells and congested capillaries are characteristic. There are a thrombocytic tendency, infarcts, fibrosis, and anemia. There is hemolytic jaundice with pigmentation. The marrow is hyperplastic[549] with evidence of accelerated hematopoiesis of all elements within the bone marrow. Unusual forms of red cells are found in specimens of bone marrow which have been prepared in formalin. The lymph glands show marked hyperplasia. A number of observers have noted phagocytosis of red cells by reticuloendothelial cells in the liver, spleen, and lymph glands. There are congestive splenic enlargement and early hemorrhage. There are siderotic nodules in the spleen. Fibrotic atrophy occurs late, as has been described by Diggs. There are hepatomegaly and some evidences of kidney degeneration. Leg ulcers are commonly seen.

The pathologic physiology of sickling was undetermined until Pauling demonstrated that the hemoglobin of sickle cell anemia (hemoglobin-S) had an electrophoretic mobility different from that of normal hemoglobin. He postulated this condition as a "molecular disease of hemoglobin."

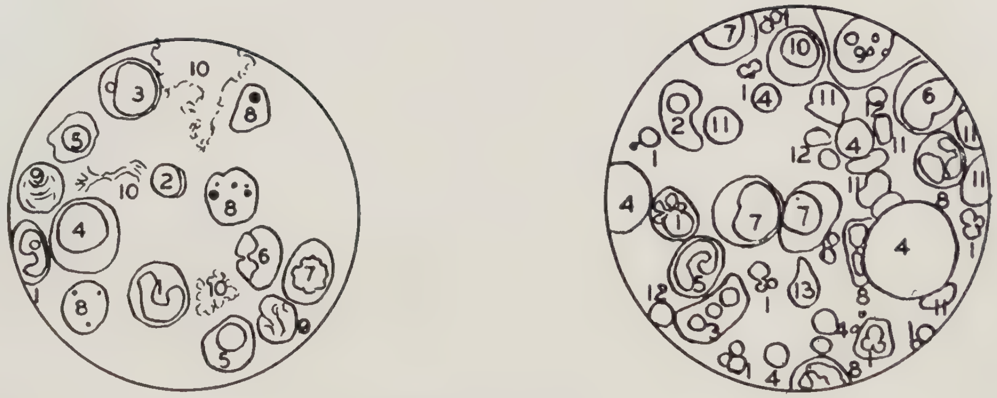
As previously stated, the globin fraction of hemoglobin-S contains valine in the beta chain in the location at which hemoglobin-A contains glutamic acid. This substitution produces profound changes so that the reduced hemoglobin-S is about 98 times more insoluble than oxygenated hemoglobin-S, whereas in comparison, reduced hemoglobin-A is only twice as insoluble as oxygenated hemoglobin-A. The low solubility and the viscous nature of reduced hemoglobin-S are responsible for the sickling phenomenon which consists of the formation of a semisolid hemoglobin gel in elongated sickle shapes called **tactoids.** This change begins at a hemoglobin-S concentration within the red cells of 10%. The tactoids form within the cell and in doing so stretch and distort the cell membrane. This process requires 3 conditions: (1) reduced oxygen, partial pressure below 45 mm., (2) a certain amount of time, 2-5 min., and (3) lowering of the pH.

Reduced oxygen tension is produced by high altitudes, flying, heart and lung disease, and anesthesia. The time factor necessary for the production of sickling is provided by the lengthened circulation time as seen in congestion and circulatory stasis. During stasis the lowering of the pH is accomplished by the accumulation of carbon dioxide. Shen and others[550] believed that a fourth factor was necessary, intravascular trauma. In the process of sickling the cells lose potassium and exchange it for sodium. The sickle cells are destroyed in the spleen.

The sickle cell trait is inherited as a mendelian dominant.

Eadie and Brown[551] have critically reviewed the literature on red cell survival studies. In sickle cell anemia it is known that when blood from a patient with the disease is transfused into a normal recipient cells disappear rapidly at first and then more slowly, with some cells living out a normal life span. The shortened survival time has been generally considered a function of the amount of abnormal hemoglobin in individual cells, those having the highest percentage of hemoglobin-S having the shortest survival time. These observers take exception to this view and suggest that random mechanical destruction plus normal senescence account for the disappearance rate in sickle cell disease. The cells in sickle cell trait have normal survival time.

As the new forms of molecular diseases of hemoglobin are uncovered, the problem of hemoglobin-S disease becomes more complex. Combinations of sickle cell with hemoglobin-C have been described, and other combinations will undoubtedly be forthcoming. This second type has a lower frequency than sickle cell disease, and Neel indicates that in 6% of Negro families in which sickle cell disease was present, the disease was due to a combination of sickle cell and hemoglobin-C. There is one gene for hemoglobin-S and one gene for hemoglobin-C. The disease is similar to hemoglobin-S disease but much milder. In the peripheral blood film target cells are prominent.



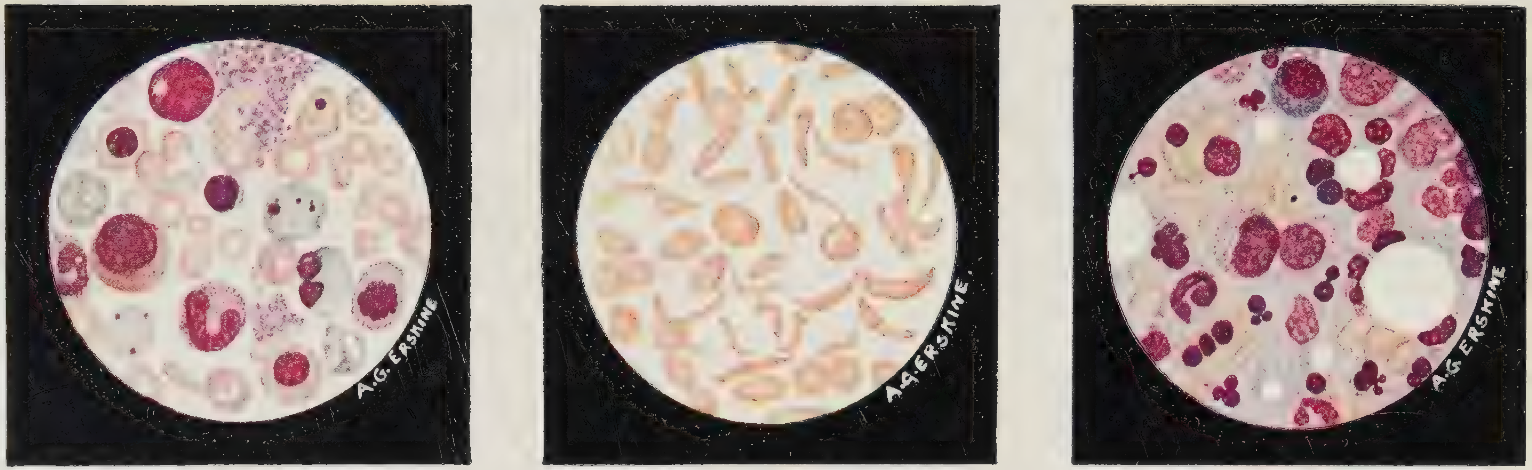
Sickle cell anemia

Blood film, regeneration

1. Stab cell
2. Lymphocyte
3. Neutrophilic myelocyte
4. Orthochromatic megaloblast
5. Orthochromatic macroblast
6. Polychromatic macroblast with karyorrhexis
7. Orthochromatic macroblast with basophilic punctation and karyorrhexis
8. Howell-Jolly bodies
9. Polychromatic macrocyte with endoglobular degeneration
10. Groups of blood platelets
11. Artifact

Bone marrow

1. Normoblasts in karyorrhexis
2. Polychromatic normoblast
3. Orthochromatic normoblast with 3 nuclei
4. Fat
5. Stab cell
6. Neutrophilic juvenile
7. Neutrophilic myelocyte
8. Segmented neutrophil
9. Myeloblast
10. Polychromatic megaloblast
11. Lymphocytes (?)
12. Nuclei of normoblasts
13. Immature nuclear structure


A.G. ERSKINE
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Sickle cell anemia

Blood film, regeneration

Wet preparation

Bone marrow

Fig. 430

The electrophoretic pattern may show hemoglobin-F in addition to hemoglobin-S and hemoglobin-C. The third type is a combination of sickle cell with thalassemia, which accounts for many of the cases of sickle cell disease in Caucasians.

Silvestroni and Bianco[552] call attention to the combination in which one parent has sickle trait and the other thalassemia trait; the offspring may have a hemolytic disease which they term microdrepanocytosis. On the basis of genetic studies in 11 families, these authors are of the opinion that the genes for sicklemia and microcythemia (thalassemia) are inherited independently of one another and are located on different chromosomes. This disease is similar to hemoglobin-S disease with moderate to severe manifestations. The hypochromia of the peripheral blood film in association with target cells and occasional sickle forms is suggestive of thalassemia-sickle cell disease. The electrophoretic pattern reveals hemoglobin-S, hemoglobin-A, and hemoglobin-F.

Some of the other combinations that have been described are sickle cell hemoglobin/hemoglobin-D disease and sickle cell hemoglobin/hemoglobin-E disease.

Hemoglobin-C disease

Hemoglobin-C is an allele of hemoglobin-A and hemoglobin-S. Hemoglobin-C is inherited as a mendelian dominant. In the **trait** no anemia is present, but target cells may be seen in the film of the peripheral blood. In the disease (homozygous form) numerous target cells are seen as well as intraerythrocytic crystals, especially after splenectomy.[553]

Ovalocytosis (elliptocytosis)

Ovalocytosis is a familial phenomenon characterized by large numbers of oval or elliptical red cells which appears as a nonsex-linked mendelian characteristic in certain individuals. The etiology is unknown. To date 75 authentic cases and 25 questionable cases have appeared in the literature. Dresbach[554] described a patient in whom every red cell was of oval shape; the blood was otherwise normal. Bishop[555] described a patient in whom 75% of the erythrocytes were oval, with the blood otherwise normal. Sydenstricker[556] reported a case of a Negro in whom 90% of the red cells were oval. Lawrence[557] reported a case of a female caucasoid, 32 yr. of age, with many oval red blood cells. Bernhardt[558] described a female caucasoid, 34 yr. of age, with oval red cells. Bernhardt termed this condition ovalocytosis. Miller and Lucas[559] have reported 9 additional cases in 4 generations. The diagnosis of elliptocytosis should not be made unless over 25% of the cells show well-developed elliptical shapes.

It must be understood that elliptical erythrocytes are not poikilocytes of any of the known anemias, including sickle cell anemia. This is a congenital anomaly compatible with an otherwise normal individual. It must be recalled that all mammals except the terrestrial family Camelidae show circular erythrocytes. Conversely, among the lower vertebrates, the red blood cells are elliptical with the single exception of the marine family Cyclostomata. Dresbach in 1902 first reported elliptical red blood cells in human beings. In the subsequent cases reported, it was found that males and females are equally affected. Ovalocytosis has been observed in a number of different nationalities. Its familial tendency was proved by Hunter and Adams[560] in 1929.

As just stated the etiology is unknown. It has been laid at the door of faulty erythropoiesis, influences of the blood plasma, the inherent quality of the cells, racial characteristics, and anoxemia, and it has been suggested that the etiology is similar to that of sickle cell anemia. Normal erythrocytes are unaltered by the plasma of persons with this anomaly. By means of autopsy, bone marrow and splenic punctures, it has been shown that these cells are not elliptical when they are formed in the bone marrow but assume that shape when subjected to the influence of some unknown constituent of the plasma or extramarrow tissues. That normal erythrocytes are unaffected by the plasma from a case of ovalocytosis suggests a second intraerythrocytic susceptibility to the extraerythrocytic influence, either or both of which may be congenital.

Miller and Lucas comment upon the fact that mechanical effects, temperature, normal blood serum, isotonic and hypotonic saline washing, picric acid, carbon dioxide, oxygen, cholesterol, etc., all failed to affect the constancy of the ellipsoid state of the red cells. Terry has shown that elliptical erythrocytes are more resistant to hemolytic agents and have a decreased fragility. He[561] has shown that these cells are heavier than round cells. Pollock and Dameshek[562] observed that potassium cyanide increased the number of elliptical erythrocytes very rapidly. Neither the marrow erythroblasts nor the reticulocytes show the oval contours.

The medicolegal point about this phenomenon is interesting; namely, within certain limits it may be used as a means of identification of both persons and paternity.

Motulsky et al.[563] divide hereditary elliptocytosis into 4 classifications, as follows:

Group I—normal round erythrocytes
Group II—oval erythrocytes
Group III—elliptical erythrocytes
Group IV—rod-shaped or cigar-shaped erythrocytes

These authors state that the predominance of Group IV cells favors a diagnosis of hereditary ovalocytosis, whereas an association of elliptical cells and other deformed cells is more indicative of symptomatic ovalocytosis such as occurs in pernicious anemia, Cooley's anemia, severe iron deficiency, leukemia, cancer, and severe anemia associated with infection. In 1 of 3 cases presented, the survival time of transfused red cells was found reduced to 45 days. This case presented other evidence of hemolysis. On the other hand, patients with normal survival times showed no hemolytic features. Motulsky et al. call attention to the similarity of hereditary ovalocytosis to other diseases characterized by deformity of the red cell and shortened life span of these cells, such as occur in sickle cell anemia, hereditary spherocytosis, and thalassemia major. Just as in these syndromes, in which there are a "disease" group and a nonsymptomatic "trait" group, ovalocytosis may likewise be subdivided. Red cell survival times in the trait group are normal, whereas in the disease group they are shortened. This survival time difference in the 2 groups is not dependent on elliptocytes; the cause for such differences remains unknown.

Molecular disease of hemoglobin accounts for the changes in sickle cell anemia, thalassemia, and other diseases, but electrophoretic studies in 2 of these 3 cases were normal and fetal hemoglobin was not increased. Motulsky et al. review the literature on symptoms and hereditary patterns in the hemolytic syndromes associated with erythrocyte shape anomaly and show the many points of resemblance between elliptocytosis and the other hemolytic groups. The hereditary pattern is considered a mendelian dominant. Splenectomy is advised in the severe condition but not in the nonhemolytic or compensated form of the disease.

HEMOLYTIC ANEMIAS DUE TO EXTRACORPUSCULAR DEFECTS

Introduction to immunohematology

Immunohematology, a term proposed by Chauffard and Troisier[564] in 1908, in recent years has attracted the attention of clinicians and investigators. The discovery of the mechanisms involved in Rh sensitization perhaps gave this field its greatest stimulus, and today the literature is replete with references to immunologic mechanisms as operative in many blood dyscrasias. Although antigen-antibody relationships have been well established for isoimmune phenomena related to red cells, knowledge of the phenomena of autoimmunity is of relatively recent origin.[565] Evans and his co-workers[566] suggested a common etiology of immunologic character for thrombocytopenia and acquired hemolytic anemia; Harrington and his group[567] brilliantly completed the picture as related to thrombocytopenia; Moeschlin and Wagner[568] have added the leukocyte to the immunologic fold. The rapidly expanding literature in the field of immunohematology contains numerous studies on clinical states in which one of the formed blood elements is predominantly involved or combinations of two or more of the elements are affected.

Immunohematology embraces all disease complexes that arise from the action of autoantibodies on the hematopoietic system of the individual and 3 major groups of diseases: (1) autoimmune hemolytic anemia due to antierythroid antibodies, (2) autoimmune thrombocytopenia due to antiplatelet antibodies, and (3) autoimmune leukopenia due to antileukocytic antibodies (and combinations of these).

Autoantibodies

Chemically, autoantibodies are globulins mainly gamma and beta but also alpha. They form in response to an antigen which is principally protein in nature but may also be a low molecular polysaccharide, either free or attached to protein. If these antibodies react best at low temperature (4° C.) and not at body temperature (37° C.), they are called cold autoantibodies. If cold antibodies are strong, their activity may well extend into the 37° C. zone. If the antibodies act best at body temperature, they are called warm autoantibodies. Each one of these antibodies may be bivalent (complete), acting best in saline medium, or univalent (incomplete), acting best in high molecular protein medium.

They are demonstrable with the Coombs technic. The hemolyzing antibodies require the presence of complement or properdin or both.

Formation of autoantibodies

Various theories have been advanced as to the circumstances which lead to the formation of antibodies against the individual's own cells.

Hapten theory: The cells are immunologically altered by the action of bacteria, viruses, enzymes, chemicals, and drugs so that they act as antigens.

Dysproteinemia theory: Osgood has postulated that autoimmune antibodies are due to a dysproteinemia in the course of which abnormal proteins are produced which happen to coat normal blood elements so that they will act as antigens. The occurrence of this type of mechanism in cirrhosis is generally accepted.

Graft versus host reaction[569]**:** The autoantibodies are abnormal antibodies produced by abnormal cells which themselves are accepted and tolerated by the body. This idea is based on experimental work of Simonsen, who produced hemolytic anemia in embryonic chicks after intravenous implantation of splenic cells of unrelated donors. In relation to symptomatic hemolytic anemia, Burnet's work is even more important, as he showed that lymphoma cells, produced by somatic mutation, survive in the host but retain their ability to produce antibodies which at times are against the patient's own red blood cells.

Isolated antigen: The theory of the isolated antigen does not apply to immunohematology since it states that substances not in direct contact with the reticuloendothelial system, such as lens tissue, thyroxin, spermatozoa, etc., may give rise to antibodies when released into the circulation.

Nature of autoantibodies[570]

Autoantibodies: Autoantibodies may be complete or incomplete agglutinins or hemolysins, acting against the individual's own cells.

Isoantibodies: Isoantibodies may be either complete or incomplete agglutinins or hemolysins. They are antibodies formed to species-specific antigens (usually blood factors) which are foreign to the individual. They may be cold or warm antibodies. They are included in this chapter because at times they lose their specificity and become "public" antibodies, directed against a large group of cells, including blood elements.

Heterogenic antibodies: These are antibodies which react with antigens different from the antigen which stimulated their production. Examples of such antibodies are those appearing in infectious mononucleosis and syphilis. Those in the first case react with sheep cells and in the second case with beef heart extract, though neither sheep cells nor beef heart muscle has an etiologic relationship to the diseases mentioned. Cold antibodies probably fall into the group of immune heterogenic antibodies, being the result of cross-reaction between, for instance, the mucopolysaccharide component of an infecting microorganism and a chemically similar substance in one of the blood cells. It must also be mentioned that there is an increasing number of reports of cold antibodies with blood group specificity.

Acquired hemolytic anemia of the autoantibody type

Clinical classification

Idiopathic hemolytic anemia: As far as known, there is no underlying disease present which could account for the occurrence of this anemia. This type of anemia occurs at any age and shows some preference for women. The cause of the disease may lie in a pathologic constitutional ability of the patient to produce a large number of various antibodies following relatively minor stimulation. An acute form of this type of anemia, which is of short duration, has in the past been referred to as **Lederer's anemia.** The clinical picture is that of all hemolytic anemias except that there is an associated tendency to thrombus formation, possibly due to release of phospholipids from destroyed erythrocytes. These thrombotic episodes are often heralded by sludging of capillary blood, visible in conjunctival and subungual capillaries. The spleen is usually enlarged.

Symptomatic hemolytic anemia: In this group fall the hemolytic anemias accompanying tumors and diseases which frequently lead to production of antierythrocytic antibodies. These conditions are chronic lymphatic leukemia, lymphomata, aplastic anemia, infectious mononucleosis, macroglobulinemia, ovarian tumors, lupus erythematosus and other collagen diseases, viral diseases such as hepatitis and viral pneumonia, and cirrhosis. It is interesting to note that multiple myeloma (high gamma globulin) is seldom associated with hemolytic anemia. In this group also fall the hemolytic anemias which follow the administration of drugs. These anemias are not synonymous with the hemolytic enzyme-deficient anemias previously discussed, which are precipitated by the administration of certain drugs.

Laboratory findings

Hematological findings

Red blood cells: The anemia is frequently macrocytic in type but differs from pernicious anemia by the presence of moderate reticulocytosis. Occasional spherocytes, siderocytes, normoblasts, and polychromatic cells are seen. The osmotic and mechanical fragility may be slightly increased, depending on the number of spherocytes. Aplastic and hemolytic crises may also occur. Autohemolysis is usually excessive. Blood samples frequently show spontaneous autoagglutination. The sedimentation rate is increased, often showing an ill-defined zone between the plasma and red cell mass produced by reticulocytes.

White blood cells: Although acute hemolysis may lead to leukocytosis, the white cell count in chronic hemolytic anemia may be depressed to leukopenic levels. Occasional erythrophagocytotic monocytes can be seen.

Platelets: Platelets are normal or depressed.

Nonhematologic findings

The findings in urine and serum are those of any hemolytic anemia (see introduction to hemolytic anemias).

Serologic findings: The antibodies involved in acquired hemolytic anemia are autoantibodies which act on the patient's own red blood cells but which frequently spread their activity to include all normal human red blood cells (isoantibody-like).

Two types of antibodies are distinguished on the basis of their in vitro characteristics: (1) warm antibodies, which act in vitro at 37° C. but are also active at lower temperatures, and (2) cold antibodies, which act best at 4° C. in vitro but extend their activity up to 28°-32° C. (room temperature) and are seldom active at 37° C.

Warm antibodies are usually of the incomplete variety and occur more frequently in hemolytic anemia than do cold antibodies. They are adsorbed by the red cells and therefore may or may not be present in the plasma, but they can be eluted from the red cells. Wiener, who calls these antibodies hyperimmune, and others showed several years ago that these antibodies may have blood group specificity and are usually linked to the Rh system (anti-**rh″**). Like most antibodies, they are gamma globulins (gamma-1 and gamma-2) and are of small molecular weight (sedimentation constant 7-S). Coated red cells are agglutinated by Coombs serum (antigamma globulin serum), and the reaction can be prevented by neutralizing the Coombs serum with small amounts of gamma globulin. On the other hand, there are sensitized red cells which are agglutinated by such neutralized Coombs serum (antinongamma globulin serum). By immune electrophoretic methods it has been shown that certain antibodies contain not only gamma globulin but also beta-2 and alpha-2 fractions. It may be that these antibodies are specific for some intermediate substance such as one of the complement components which under the influence of the antibody are bound to the red cell which then secondarily becomes coated with a gamma globulin type of antibodies.

Cold antibodies are usually complete antibodies and are frequently nonspecific in nature. Wiener,[571] who calls these antibodies simple, showed that some cold antibodies have blood group specificity. The particular antigen involved is named antigen **I**[572] and the antibody anti-**I**. The immune antibody—and this applies generally—is at first specific for a single antigen but later also affects related antigens.

The term nonspecific implies that the etiologic antigen has not been identified, but it may also connote that it acts on an antigen common to all red blood cells. Thus while cold antigens may be specific, they frequently act on all red blood cells and sometimes even on heterogeneous red cells. Electrophoretically, the cold antibodies are gamma-1 (beta) globulins with a sedimentation constant 16-19-S (macroglobulins). They act best at 0°-4° C., but they may have a wide thermal range and extend up to 28°-32° C. If they are hemolytic, they interact with complement best at an acid pH of 6.3. A special type of cold hemolysin is Donath-Landsteiner cold hemolysin which unites with the red blood cells best at a temperature of 0°-4° C., but hemolysis does not occur until the temperature rises to over 15°-40° C. The latter hemolysin is called bithermic in contrast to the nonthermic hemolysin which acts best at a pH of 6.3. In the latter case both reactions, binding of the complement and lysis of the red cells, occur at the same temperature.

Laboratory investigation of warm antibodies

Direct antiglobulin test (direct Coombs test): This test represents a gamma globulin-antiglobulin reaction, as human erythrocytes sensitized by gamma globulin antibodies are agglutinated by an antihuman globulin serum prepared in rabbits. This test should be performed at 4° C., at room temperature, and at 37° C., always with appropriate controls. For the detection of warm antibodies only, performance of the test at room temperature and at 37° C. suffices. The sensitivity of this test can be improved by using enzyme-treated cells (trypsin, papain, albumase, etc.). A positive test (agglutination) proves the presence of an antibody on the surface of the patient's erythrocytes (for details of technic see discussion of blood groups).

Agglutination of cells suspended in albumin: Patient's washed cells are transferred to 20% albumin to produce a 5% suspension. After 1-2 hr. incubation at 37° C. sensitized cells will undergo autoagglutination.

Agglutination of trypsinized cells: Patient's cells are washed, enzyme treated, and after incubation in albumin (see above) will frequently show agglutination.

Preparation of trypsin stock solution:

1. Prepare a 1% stock solution trypsin powder in phosphate buffer, pH 7.4.
2. Filter through Whatman No. 1 filter paper and distribute into small cubes and store frozen in the freezer.
3. Add 1 part stock trypsin solution to 10 parts 2% saline suspension.
4. Incubate in water bath at 37° C. for 10 min.
5. Wash the cells 3 times with saline.
6. Drain saline and add fresh saline to make a 2% cell suspension.
7. Suspend 2 drops trypsinized cells in 2 drops albumin.
8. Incubate at 37° C. for not longer than 1 hr. and examine for agglutination. Do not centrifuge.

It must be pointed out that there are antibodies which can be detected by the indirect Coombs technic but which fail to react with trypsinized cells (**K**, **Fy**^{a}, etc.).

Nongamma globulin antiglobulin test (gamma globulin neutralization test or complement antiglobulin test): There are some autoantibodies that do not react with antigamma globulin Coombs serum, such as anti-**H**, anti-**Jk**^{a}, and anti-**Fy**^{a}. These antisera are apparently specific for human complement (C^{1}) rather than for gamma globulin. The direct Coombs test can be inhibited by the addition of human gamma globulin. On the other hand, the addition of human gamma globulin fails to inhibit a positive nongamma globulin antiglobulin test in which complement is involved. This test is inhibited by the addition of human alpha and beta globulin. In the execution of the test, antiglobulin serum is partially neutralized with human gamma globulin, and the direct Coombs test is performed utilizing the partially neutralized serum.

Indirect antiglobulin (Coombs) test: The purpose of this test is to detect antibodies in the patient's serum by incubating Group O Rh-negative cells with the patient's serum. If the patient's serum contains incomplete antibodies, they will attach themselves to the red blood cells, thus sensitizing them. The direct Coombs test is then performed on these in vitro sensitized cells. The use of albumin or enzyme-treated cells will increase the sensitivity of this test but makes several control procedures necessary.

Specificity of antibodies: After it is established that warm antibodies are present in the serum, their specificity can be established by performing the albumin and the direct Coombs test with the serum on a panel of cells containing most of the known antigens.

Laboratory investigation of cold antibodies

The tests employed are essentially the same as for the warm antibodies but require certain modifications.

It should be mentioned that rouleaux formation can at times be mistaken for cold agglutination though microscopically it is usually easy to differentiate from true agglutination. Rouleaux formation can be disturbed by pressing on the cover glass, and it is usually diminished by adding saline or lecithin to the serum. Whereas cold agglutination usually disappears at 37° C., rouleaux formation may become even more pronounced.

Test for cold agglutinins:

1. Collect 4 ml. blood in 1 ml. 3.8% sodium citrate. The plasma dilution will be 1:4.
2. Set up 4 sets of 7 Kahn tubes, adding 0.5 ml. saline to each tube.
3. To the first tube of each set, add 0.5 ml. 1:4 diluted serum, mix, and then transfer 0.5 ml. to the next tube, etc. Each set of tubes should also contain 1 control tube.
4. To each tube then add 0.5 ml. 2% suspension of Group O cells washed in saline.
5. Allow these sets to remain at the following temperatures: 4°, 16°, 22°, and 37° C. for 12-20 hr. and then examine for agglutination.

Test for cold hemolysin: See explanation of acid hemolysis test in discussion of paroxysmal nocturnal hemoglobinuria.

Test for hemolysis:

1. Divide patient's blood and normal control blood into 2 test tubes each and allow to clot.
2. Place 1 tube of each set in an ice bath (3°-4° C.) for 20 min. and then transfer to a 37° C. water bath for 1 hr.
3. Examine for hemolysis.

The second tube of each set is left at room temperature.

Antiglobulin test: The antiglobulin test may be positive at room temperature in the face of a high-titered incomplete cold agglutinin associated with hypergammaglobulinemia due to liver disease, infection, collagen disease, etc. These incomplete antibodies may be distinguished from the complete cold agglutinins by using neutralized Coombs serum which does not inhibit the reaction of true cold agglutinins which interact with complement (see discussion of cold agglutinins and of gammaglobulin neutralization test). Further 7-S gamma globulin can be eluted from the red cells, and these eluates will react with other red blood cells, whereas nongamma globulin antibody eluates will not do so.

Chronic and acute hemolytic anemias due to the presence of cold agglutinins

Congenital and acquired syphilis[573] are rarely accompanied by chronic hemolytic anemias due to cold agglutinins. Viral pneumonia[574] frequently leads to an acute hemolytic anemia due to the presence of abnormally high titered cold agglutinins. A paroxysmal, nonsyphilitic form of hemolytic anemia due to cold agglutinins has also been described. In all these cases, peripheral vascular occlusion leading to gangrene is an important complication. The serum may contain cryoglobulins. Cryoglobulins are tested for by simply exposing the serum to 4° C. for a period of 12 hr. to see whether it gels at that temperature.

Hemolytic anemias due to isoantibodies (erythroblastosis fetalis and hemolytic transfusion reactions)

Erythroblastosis fetalis

This is often a fatal condition which has been the subject of considerable investigation. It was originally described as being due to an intragroup hemolytic reaction incident to the mating of an Rh-positive father with an Rh-negative mother. It has been well described clinically by Buhler et al.,[575] who reported on this disease in dizygotic twins. The clinical manifestations and their development will depend on whether the predominate pattern be evidenced by edema or icterus or simply by the blood findings of anemia and an increased number of nucleated red blood cells. Hydrops when associated with either of the other two is fatal, and the infant is either stillborn or dies immediately after birth. Jaundice is absent at birth but appears within a few hours to several days, depending on the severity of the disease process. Potter and Adair[576] point out that neither the anemia nor the increased nucleated red blood cells alone are sufficient for a diagnosis of erythroblastosis fetalis, and many cases so diagnosed with subsequent recovery are probably erroneous. Buhler et al. record the symptoms in infants as follows: lethargy and exhaustion occur; symptoms may develop rapidly, depending upon the rate of blood destruction; petechial hemorrhages may appear; the heart is often enlarged; the pulse is rapid and weak; usually fever is not present; the liver and spleen are enlarged; and due to deposition of bilirubin and heme in localized areas of the brain, a syndrome called **kernicterus** may develop which may quickly cause death. McKinley[577] records the early symptoms of kernicterus as convulsions, neck retraction, respiratory paralysis, and drowsiness.

Danis and Anderson[578] consider the syndromes of congenital hydrops, erythroblastosis fetalis, icterus gravis, and anemia of the newborn, one condition, i.e., a condition known as icterus gravis neonatorum.

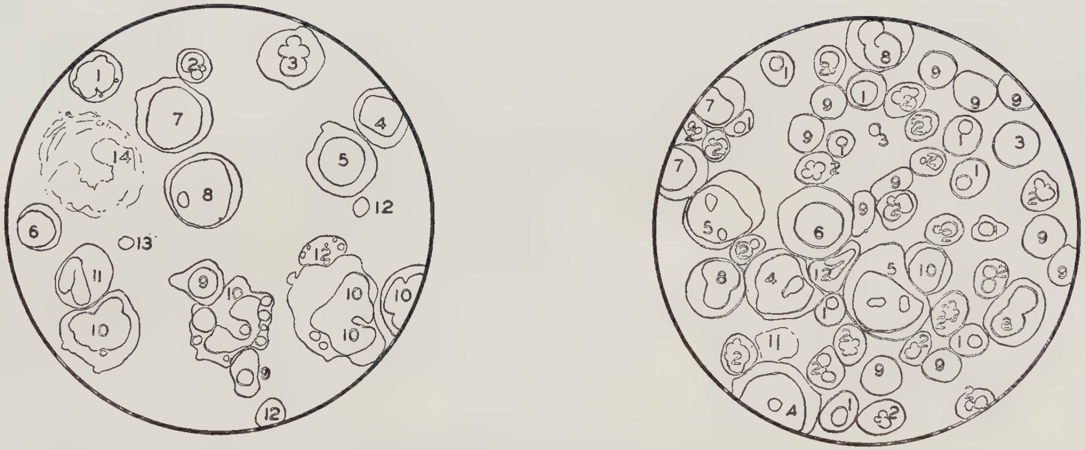
Laboratory findings,[579-583] are those of a macrocytic hyperchromic, hypocythemic anemia associated with a hemolytic type of jaundice. There is an increase in the nucleated red cells. Hemoglobin varies from 25-60%. The red cell count is from 1,000,000-3,000,000. Reticulocytes are increased to from 15-20%. Poikilocytosis and anisocytosis are present. Leukocytosis is often marked, with an increase in the young white forms. The erythrocytes in erythroblastosis due to Rh incompatibility have been shown to have a decreased oxygen-carrying capacity and decreased glycolytic rate.[584]

Platelet counts are inconstant, and fragility tests are variable. Bleeding time is often prolonged; the coagulation time is not affected. The urine contains bilirubin. The stools may be acholic but more often are not.

Pathologic features are a universal hydrops, edematous tissues, ascites, hydrothorax, and hydropericardium if the infant dies of heart failure due to the anemia. If heart failure is absent, hemolytic jaundice is the outstanding finding.

Microscopically, the tissues show extramedullary hemopoiesis with islands of erythropoiesis occurring in the liver, spleen, adrenals, and kidneys and occasionally in other organs. The bone marrow is hyperplastic. Bile deposition is noted in the nuclear areas of the brain. The placental pathology is characteristic, i.e., increase in size, grayish or icteric color, and friable, edematous villi. Microscopically the villi are enlarged, and the vessels are filled with nucleated red cells.

Erythroblastosis fetalis is a hemolytic anemia of the fetus and newborn due to the action of antierythrocytic isoantibodies transferred across the placenta from the mother to the fetal circulation. The hemolytic anemia leads to jaundice because of the increase in indirect bilirubin (toxic!) and may also lead to heart failure (edema, hydrops). The sensitization of the mother can be produced by any antigen which she lacks and is in no way confined to the Rh system. Though erythroblastosis due to Rh incompatibility was the first form to be described, we now believe that A-B-O erythroblastosis is twice as common but is usually not clinically as severe and dangerous as Rh erythroblastosis. The other blood factors are of much less importance in the pathogenesis of erythroblastosis. If the sensitization of the mother is produced by pregnancy, usually the first pregnancy will not then be affected. Sensitization during pregnancy follows the release of fetal red blood cells into the maternal circulation, especially during delivery. Only a very small amount of fetal blood is needed to produce such sensitization. Operative obstetrical procedures such as cesarean section, abortion,



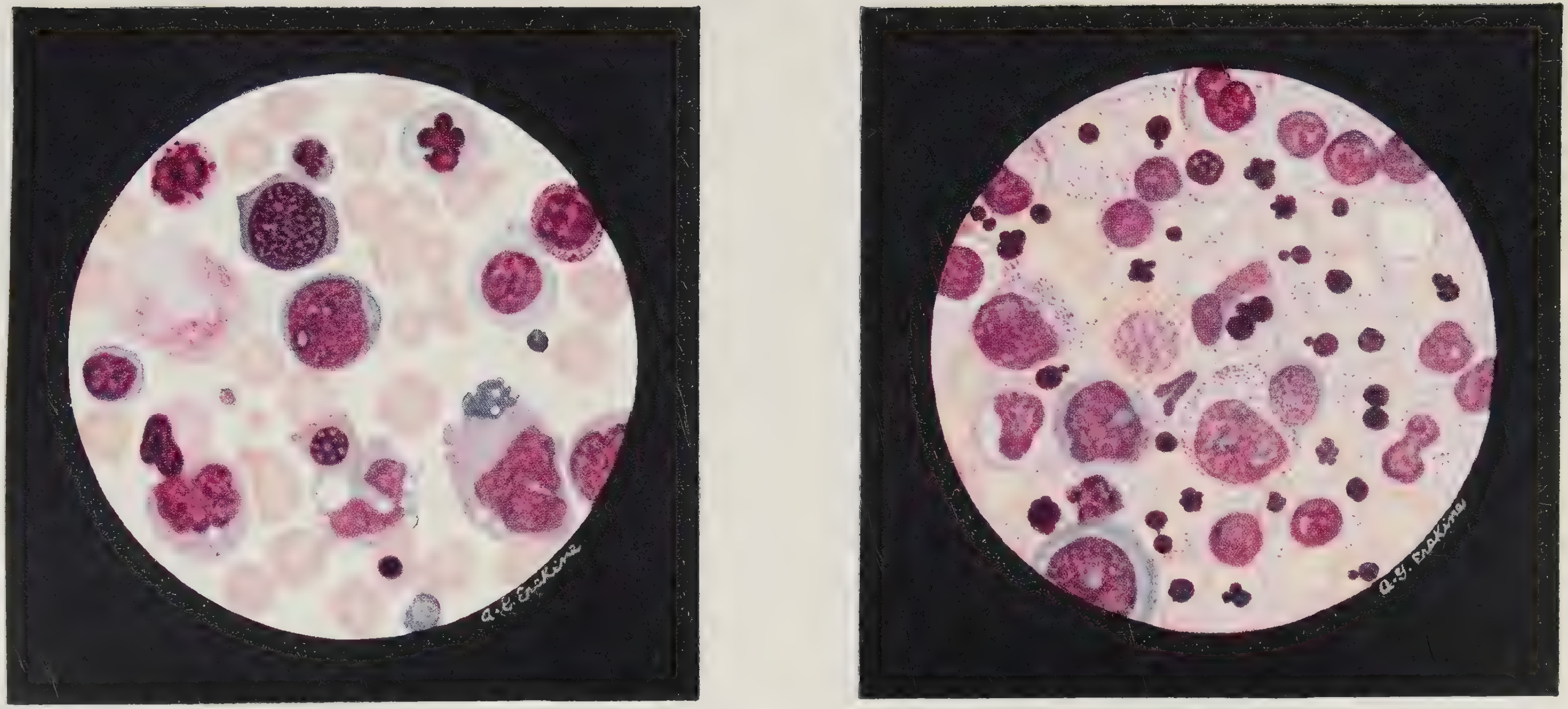
Erythroblastosis fetalis—fatal case

Blood film (Giemsa stain; ×950)

1. Basophil
2. Normoblast in karyorrhexis
3. Macroblast in karyorrhexis
4. Neutrophilic myelocyte
5. Large lymphocyte
6. Small lymphocyte
7. Polychromatic megaloblast
8. Lymphoblast
9. Normoblast
10. Monocyte
11. Stab
12. Polychromatic erythrocyte
13. Blood platelet
14. Fragmentary nuclear structure
    Note vacuoles in monocytes

Bone marrow (Wright-Giemsa stain; ×950)

1. Normoblast
2. Normoblast in karyorrhexis
3. Fat
4. Lymphoblast (?) or myeloblast, one with Auer rods
5. Promyelocyte
6. Eosinophilic myelocyte
7. Neutrophilic myelocyte
8. Neutrophilic juvenile
9. Lymphocytes (?)
10. Polychromatic macroblast
11. Fragmentary nuclear structure
12. Neutrophilic stab cell



Erythroblastosis fetalis—fatal case

Blood film

Bone marrow

Fig. 431

etc. tend to increase the incidence of sensitization. If the sensitization is produced by the administration of parenteral blood, the firstborn child may develop erythroblastosis fetalis.

Frequency of sensitization: Many factors determine the incidence of erythroblastosis fetalis (see discussion of blood groups).

1. Contact with a foreign antigen is not synonymous with sensitization. Not all women exposed to sensitization will actually develop antibodies, as apparently constitutional factors determine the ease with which they are produced.

2. Not all antigens are equally antigenic. In the Rh group in order of decreasing potency, the antigens are **Rh_0**, **rh′**, **hr′**, **rh″**, and **hr″**, and in the other blood groups they are **A**, **B**, **K**, **Jk^a**, **Fy^a**, **k**, **s**, and **Wr^a**. References are given to rare antigens causing erythroblastosis (**M**,[585] **U**[586]).

3. The A-B-O type of fetal red blood cells may affect the incidence of erythroblastosis. On one hand, the A-B-O type of the fetal cells may be responsible for the production of so-called A-B-O erythroblastosis. On the other hand, it influences the frequency of Rh sensitization and thus the frequency of Rh erythroblastosis. In cases of fetal-maternal Rh incompatibility, if the fetal cells are A-B-O compatible with the mother cells, they are not destroyed within the mother's circulation and may thus accumulate to reach sensitizing levels. If the fetal cells circulating within the mother's blood stream are A-B-O incompatible with the mother's blood, they are probably rapidly destroyed in the maternal circulation and are thus prevented from reaching sensitizing proportions.

4. As far as Rh erythroblastosis is concerned, it is important to know whether the Rh-positive husband of an Rh-negative woman is homozygous or heterozygous for the Rh factor involved. The more frequently and persistently an Rh-negative woman is exposed to sensitization, the more likely she is to eventually become sensitized. Thus a woman mated to a homozygous male is more likely to become sensitized than one mated to a heterozygous male.

5. The secretor state of the baby is important. Nonsecretor babies are not as well protected against the action of maternal isoantibodies as are secretor babies who are able to neutralize the antibodies before they reach the red blood cell. The secretor state is inherited but is independent of the blood groups and is most easily determined by testing the saliva for A and B blood group substances.

Erythroblastosis due to Rh incompatibility and its laboratory diagnosis

The prenatal investigation of any patient should include determination of the A-B-O blood group and of the Rh factor. If she is Rh negative, the presence of an anti-Rh antibody and its titer should be determined at about 6-7 mo. of gestation, again a few weeks before expected delivery, and finally a few weeks after delivery. This procedure should be followed with each pregnancy (including the first). The antibody titer is important in the discovery of erythroblastosis of the firstborn and is of importance if the titer is rising. (Always place the originally titrated serum in the freezer and retitrate it in conjunction with the second or third specimen). The actual level of the titer may bear little relationship to the severity of erythroblastosis, but it serves as a warning signal. We like to screen all multiparous women and all women who have a history of having received a transfusion for atypical antibodies (Hemantigen*), irrespective of their Rh factors. If atypical antibodies are found an attempt should be made to identify them (Panocell*).

The result of the action of the transmitted antibodies on the fetal red blood cells is their destruction and the liberation of indirect acting (nonconjugated) bilirubin which is insoluble in water and is transported in the blood stream linked to serum albumin. The poorly developed hepatic enzyme system of newborn and premature babies is unable to conjugate all the oncoming direct bilirubin to glucuronic acid. The nonconjugated bilirubin is not excreted by the liver or the kidneys and therefore accumulates in the tissues. It is associated with the "absolute heme pigment" of Abelson and Boggs, for the determination of which these authors describe an easily performed method.[587]

If an Rh incompatibility exists irrespective of the result of the titer determination, the newborn baby's red blood cells should be tested for the A-B-O and Rh types and for sensitization by the Coombs technic.[588] The baby's venous blood is at times preferable to cord blood, which may show a lower bilirubin titer and may also be contaminated with cord jelly. In rare cases a false negative

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*Knickerbocker Laboratories, New York, N. Y.

Coombs test may be produced because of antibodies with blocking action, and it should also be remembered that the baby may actually be **Rh_0** negative and that the erythroblastosis may be due to some other factor than Rh. The mother's blood should be tested for atypical antibodies.

The level of the indirect reacting bilirubin in the baby's serum is measured at birth to obtain a base line and again within 6 hr. postpartum. In a newborn infant almost all the bilirubin measured is of the indirect variety. The following bilirubin levels are indications for exchange transfusions, according to Allen and Diamond[589]: birth level of 7 mg., a rise of 1 mg./hr., and a level of above 20 mg.

A-B-O erythroblastosis and its laboratory diagnosis

A-B-O erythroblastosis[590, 591] occurs mainly in type O women who apparently more readily develop high titer anti-**A** and anti-**B** antibodies than do type A and type B women. Wiener postulates that group O serum contains in addition to anti-**A** and anti-**B**, a third agglutinin called anti-**C**, directed against a factor **C** contained in agglutinogens A and B. Anti-**C** antibody is presumed to be able to cross the placental barrier more readily than anti-**A** and anti-**B**. On the other hand, one can assume that anti-**A** and anti-**B** antibodies are not homogeneous but are made up of multiple fractions. Thus Fischer on the basis of differential agglutination with anti-**A_1** shows that in newborn group A infants the percentage of agglutinable cells varies widely from one individual to another. He believes that this is due to the fact that the Group A infant may contain group A_1 and group A_2 cells and that the mother's antibodies act only against the A_1 erythrocytes.

Fascinating, though not proved, is the question of immunologic tolerance and its relationship to the incidence of erythroblastosis. Animals exposed to antigens in fetal life may fail to develop antibodies against this particular antigen in later life. One can thus assume that a group O fetus of a group A woman is exposed to A substance in utero and may therefore in later life not so readily form anti-**A** antibodies.

The jaundice is usually light, and the direct Coombs test on fetal red blood cells is usually negative. In the mother's serum the titer of the naturally occurring isoantibodies is elevated and is often exceeded by the titer of an immune antibody. It may be detectable after neutralization of the isoantibodies by Witebsky substances (blood group substances A and B).

The hemolytic anemia is usually moderate and characteristically the peripheral blood film shows the presence of spherocytes. On occasion, normoblasts are seen.

Tests for A-B-O erythroblastosis

Witebsky slide test:

1. Divide a slide into 3 compartments and place it on a heating viewing box.
2. Into the first compartment, place 1 drop mixture 1 part normal adult serum to 2 parts bovine albumin.
3. Into the middle compartment, place 1 drop normal adult AB serum and into the third compartment place 1 drop normal saline.
4. Add 1 drop heavy suspension cord blood to each solution.
5. Mix and examine for agglutination not longer than 2 min.

Cord red blood cells of infants with A-B-O erythroblastosis are agglutinated most strongly in the normal AB serum and less strongly in the albumin-serum mixture. The reverse is frequently true of Rh erythroblastosis.

Control: Set up a similar series of solutions and add 1 drop heavy suspension normal blood.

Witebsky neutralization test for anti-A and anti-B immune antibodies:

1. Prepare unneutralized serum by separating serum from clotted whole blood.
2. Prepare neutralized serum. To 1 ml. serum add 0.1 ml. concentrated A and B substances; allow to stand for at least 1 hr.
3. Prepare 2% cell suspension in saline of A and B cells which have been washed once. Also prepare 2% suspension of A and B cells in 20% albumin.
4. Titer of isohemagglutinins:
    - (a) Prepare 2 sets serial dilutions of unneutralized serum in normal saline diluting from undilute to 1:1024. Each tube should contain 0.1 ml. serum dilution.
    - (b) To 1 set of tubes add 0.1 ml. 2% saline suspension of A cells and to the second set of tubes add 0.1 ml. 2% suspension of B cells.
    - (c) Do not incubate. Centrifuge at 1000 r.p.m. for 1 min. and read the isohemagglutinin titer.

Examination of neutralized serum:

1. Using neutralized serum prepare 2 sets of serial dilutions in saline from undiluted to 1:16. Each tube should contain 0.1 ml. of serum dilution.
2. To 1 set add 0.1 ml. 2% A cells suspension and to the second tube 0.1 ml. B cells suspension.
3. Mix, centrifuge, and read.

If the isohemagglutinins have been neutralized, no agglutination will occur, or it will be only weakly positive in the first tube.

Titer of immune antibodies:

1. Using neutralized serum, prepare 2 sets of

serial dilutions of serum in 20% albumin. Each tube should contain 0.1 ml. serum-albumin mixture. The dilution should be from undiluted to 1:128.

2. To the first set add 0.1 ml. 2% suspension of A cells in 20% albumin and to the second set add 0.1 ml. 2% suspension of B cells in albumin.
3. Incubate for 30 min. at 37° C.
4. Shake, centrifuge at 1000 r.p.m. for 2 min., and read.

If an immune antibody is involved, it will show a high titer in the high protein test.

Ion exchange column chromatography: Kochwa[590] points out that the distinction between so-called natural agglutinins, which do not cross the placenta and so-called immune agglutinins which do cross the placenta cannot be maintained, as all agglutinins are basically immune in type. (Natural antibodies are immune in origin due to contact with plants, seeds, and foods containing A and B substances.)

Kochwa was able to separate the isoagglutinins which crossed the placenta from the remaining serum proteins by ion exchange column chromatography.

Treatment of erythroblastosis fetalis[585, 586, 591]

A single laboratory finding on the infant's blood, such as a high or low hemoglobin, a high or low bilirubin level, erythroblastemia, or reticulocytosis, has no diagnostic value. A positive Coombs test is diagnostic; a negative one does not rule out erythroblastosis. A premature infant may have a low cord bilirubin titer and a sharp rise later on. It may be that Boggs absolute heme pigment determination will prove to be a better criterion of the severity of erythroblastosis than bilirubin levels.

The treatment of choice at the present time is exchange transfusion and therefore involves the laboratory. The blood used for the exchange must be compatible with the mother's blood and should be of the same A-B-O group as the infant and of course must be free of the antigen to which the mother has been sensitized. The newborn infant does not have globulins or antibodies of its own—they are all supplied by the mother—it therefore lacks isoantibodies.

Since the infant's A and B antigens are weak and reverse typing is not informative, it is at times difficult to be sure of the infant's type. It is therefore advisable and safe to administer group O Rh-negative blood except in the rare case of erythroblastosis due to **hr′** sensitization. In emergency, the mother's red blood cells in saline suspension can be used. Exchange transfusions carry the same risk as all other transfusions, such as the danger of hyperpotassemia, hypocalcemia, citrate intoxication, overloading of the circulation, thrombocytopenia, and hypoprothrombinemia. The use of heparinized blood (instead of A-C-D blood) has been suggested, followed by neutralization of heparin with protamine sulfate and the administration of vitamin K_1 and calcium glucuronate. Large doses of vitamin K have been shown to increase the serum bilirubin level in young infants.

Hemolytic anemia due to incompatible blood transfusions

There are 2 possibilities. First, the donor's erythrocytes are not compatible with the recipient's blood, in the plasma of which are either isoantibodies or immune antibodies acting on the donor's red blood cells. This process, which is the most common occurrence in transfusion reactions, does not lead to anemia, as the relatively small number of donor's cells is rapidly destroyed. It may, however, lead to transfusion reactions, shock, and lower nephron nephrosis.

The second possibility is that the antibodies present in the donor's blood are acting against the recipient's red blood cells.

TOXIC HEMOLYTIC ANEMIAS

Toxic hemolytic anemias follow contact with foreign substances such as bacterial and viral toxins, drugs and medications, and poisons of industrial, plant, and animal origin.

Hemolytic anemia following infection

Viral infections

Although a hemolytic anemia may accompany or follow almost any viral infection, it most commonly follows in the wake of viral pneumonia. A sudden hemolytic episode may occur at the time the patient has about recovered from the pneumonia. The appearanec of the anemia is associated with a marked rise in cold agglutinins. A hemolytic anemia may be one of the first symptoms of infectious mononucleosis,[593] and it may accompany infections with the virus of measles, smallpox, and Coxsackie virus.

Hematologic findings: The anemia is usually macrocytic in type, and the peripheral blood film contains a goodly number of spherocytes, characteristic of so many hemolytic anemias and responsible for the increased osmotic fragility. The other features of hemolytic anemias also present are polychromasia, reticulocytosis, occasional siderocytes, and normoblasts.

The immune destruction may not only involve the red blood cells but also may spread to platelets and to white blood cells to produce thrombocytopenia and leukopenia. Usually moderate leukocytosis accompanies the acute hemolytic episode. Frequently autoagglutination of the patient's red blood cells can be observed.

Bacterial infections

Hemolytic anemias have been described in infections caused by hemolytic streptococci and staphylococci, such as acute and subacute bacterial endocarditis and puerperal infections. In *Clostridium* infections following injury or abortion severe hemolytic anemia may occur. Hemolytic anemias also accompany typhoid fever and cholera.

Parasitic infections

Only a relatively small group of parasites characteristically lead to the production of severe anemias, either by their action on the red blood cells or the hematopoietic system or both or by interference with intestinal absorption and production of intestinal injury (chronic blood loss). A few anemias are hemolytic.

Parasites which affect red blood cells and the hematopoietic system (hemolytic anemias) are the following:

Bartonella (Oroya fever)
Plasmodium (malaria, blackwater fever)
Leishmania (kala-azar)
Ancylostoma duodenale; Necator americanus (ancylostomiasis)
Diphyllobothrium latum
Entamoeba histolytica (amebiasis)

It must be stressed that at times infestations with other types of amebae, cestodes, or nematodes produce moderate degrees of hypochromic anemias.

Blood picture in bartonellosis (Oroya fever): The blood picture in bartonellosis, according to Groot[594] shows a rapid, progressive anemia. Erythrocyte counts of 1,000,000/c.mm. are common. The rate of decrease is rapid, with as many as 300,000 red cells/c.mm. lost in 24 hr.

The volume, diameter, and thickness of the erythrocytes are greater than normal. The mean corpuscular hemoglobin concentration is usually low but may be normal. Hyperbilirubinemia is often observed but is not constant. The bone marrow is regenerative, with large numbers of reticulocytes and normoblasts in the peripheral blood, the number of such elements being proportional to the seriousness of the anemia. Megaloblasts are often found.

Bartonellae are found in the red blood cells, the number varying. At times, 99% of the red blood cells are infected, and often it is possible to find 10-15 organisms in a single cell although these are extreme cases. Basophilic erythrocytes and macrocytes are usually less infected. Reticulocytes are seldom infected unless the number of bartonellae in the blood is very high. Normoblasts are seldom infected except in rare cases. Bartonellae are sometimes seen in the cytoplasm of monocytes.

At the beginning of the disease there is leukopenia although the number of white blood cells is normal in uncomplicated cases.

There is no typical differential count. At times either a very slight lymphocytosis is noted or more commonly polymorphonucleosis.

During the period of anemia there is an increase in young polymorphonuclear cells, etc., in the myeloid series.

The sedimentation rate is greatly increased, 100-172 mm. in 1 hr. being noted in the Westergren test.

Autoagglutination phenomena are rare.

According to Schilling this is the only acute infection which causes a severe anemia of the megalocytic type.

Malaria: The general blood changes in malaria* are as follows. Anemia is always present. It is caused both by direct destruction of erythrocytes due to the development of the parasites within them and also by toxic influences. There is oligocytosis, a lowered hemoglobin content, and a lowered color index. Polychromasia is usually marked, and coarse basophilic punctation is a frequent blood finding. In severe anemia normoblasts are found. There may be nuclear spheres, marginal granules, and other nonspecific indicators of anemia. Parasites have been observed in polychromatic and stippled erythrocytes and even in normoblasts.

Malaria pigment may frequently be found in phagocytes, usually in large monocytes (pigmentophages) although we have seen them in all 4 types of neutrophilic cells. This pigment is yellow or brown and light refracting. It is usually arranged in characteristic clumps. It originates from devoured or phagocytized parasites and is important in detecting malarial infection when no parasites are found. It is demonstrable not only in blood spreads but also in thick drop preparations.

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*See section on parasitology.

During the attack, there is irritation leukocytosis with marked regenerative shift, neutrophilia, and decrease in the number of eosinophils. **During reduction of fever** and **shortly following this,** there are leukopenia, marked **monocytosis** and **relative** lymphocytosis, with slight eosinophilia. In **severe relapsing cases** there is a continuous stab shift. In **chronic cases,** low white counts, monocytosis, and lymphocytosis are found. The white count rises rapidly, but only temporarily, shortly after an open or latent attack. In this condition parasites are scarce. If there is a continued shift with monocytosis after treatment, it is thought to indicate recurrence. In clinically suspected malaria in which no parasites are found in the blood, one is led to the diagnosis of malaria if polychromasia is present as 2 or 3+ in the thick drop, if basophilic punctation is found, and if lymphocytes and monocytes are markedly increased, especially if quinine has already been administered.

All varieties of malaria show similar blood pictures, but acute falciparum malaria may show a higher neutrophilia with a regenerative shift to myelocytes.

In **parasite-negative cases,** the administration of 0.5-1 ml. 1% epinephrine may aid in the demonstration of the parasites. Other methods that have been used are repeated ultraviolet ray exposures and severe physiotherapy with alternating hot and cold douches. Thick drop should be examined repeatedly at intervals of several hours.

In tropical malaria, there may be both hyperleukocytosis and a left shift. The typical hemogram of **falciparum malaria** is shown in Table 251.

In the thick drop preparation there are marked polychromasia and basophilic punctation and many ring forms of *Plasmodium falciparum.*

In this disease there is severe hyperleukocytosis with comparatively high lymphocytosis and an increase in monocytes. There are no eosinophils, and there is a marked toxic polychromasia.

Blackwater fever: Blackwater fever is a hemolytic complication of falciparum malaria which leads to severe hemoglobinemia and hemoglobinuria and is subject to all previously discussed renal complications. The course is unknown but may be related to an unusual sensitivity of the red blood cells to lysolecithin.

In blackwater fever there is a marked decrease in the number of erythrocytes and in hemoglobin content. This is soon followed with improvement by a very marked regenerative normoblastic blood picture. Upon continuation or onset of new attacks, megaloblastic or aregenerative (reverse) blood pictures occur. This is an unfavorable symptom. Deaths have occurred in blackwater fever due to suppression of erythropoiesis. Parasites usually disappear temporarily during an attack of blackwater fever.

Toxic hemolytic anemias following poisoning

Lead poisoning

Hematology of lead poisoning

The effects of alimentary, respiratory, or cutaneous contact of lead with the human body lead to a definite change in the microscopic picture of the red blood cells; in the main these are the appearance of the classic basophilic punctation and a mild degree of polychromasia. These changes in the blood are the first and most striking signs of lead poisoning in man.

According to Aub and others, Laennec[595] first recognized lead anemia in man. Exposure of experimental animals to lead is followed by marked anemia. This was demonstrated in rabbits by intramuscular injection by Carcanaque and Maurel[596] and after oral administration by Key.[597]

Two causes of this anemia have been described: marked destruction of blood or a lesion in the bone marrow with consequent deficient blood formation. According to Stockman and Charteris,[598] and Raimondi,[599] bone marrow of rabbits showed a marked preliminary increase of leukoblasts with disappearance of fat, followed later by definite gelatinous degeneration. Wolff[600] described a case of severe lead poisoning in which at-

Table 251

Basophils	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*
0	0	0	18	32	20	18	12

tempts to regenerate blood were followed by deficient hematopoietic function, as seen in the bone marow. Meillére,[601] Schnitter,[602] and Sellers[603] thought that lead definitely injured both blood cells and bone marrow. Bouchard[604] and later Rauch[605] and others attributed the primary appearance of anemia to abnormal destruction of the cells in the circulation even though degeneration of the bone marrow may occur later. Evidence for this is diverse. Early lead poisoning shows hyperplasia of the bone marrow[598] with many nucleated red cells in the circulation which seems to indicate regeneration. Key[597] demonstrated that within 24 hr. after ingestion of 1 gm. lead the number of circulating erythrocytes in rabbits decreased more than 20%, with the appearance of many nucleated red cells. Meillére showed a definite increase in urinary excretion of iron, largely derived from erythrocytes, in the onset of lead anemia. J. W. S. Brady[606] in Aub's laboratory demonstrated that slow intravenous injection of lead acetate dissolved in saline caused marked hematuria and very marked increase in excretion of bile pigments from the common duct. Chester Jones[607] obtained similar evidence in human cases. He showed that both the blood plasma and bile obtained by duodenal drainage from several cases of plumbism contained very high concentrations of bile pigments. These facts indicate marked peripheral destruction of blood due to ingestion of lead.

Erythrocytes in lead poisoning show increased resistance to hemolysis in dilute saline solution. Malassez[608] reported in 1873 that not only is anemia a symptom of lead poisoning, but also that the erythrocytes are larger and more fixed than normal. Agasse-Lafont and Heim[609] found slightly increased globular resistance in lead poisoning. Von Liebermann[610] and later Orban[611] and Hayhurst[612] demonstrated this. Fici[613, 614] studied this reaction in vitro in 1921 and found that lead acetate exerts a striking effect on the hemolysis of red cells in hypotonic saline solutions, varying with concentration of lead and with temperature.

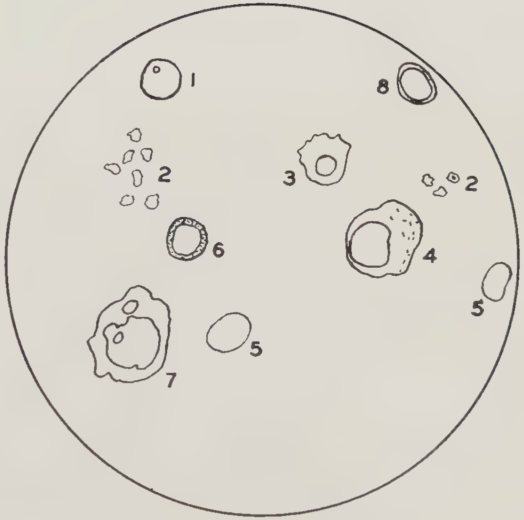
The basic defect in lead poisoning is that lead prevents the incorporation of iron into the protoporphyrin molecule so that abnormal red cells with basophilic stippling are produced. Bessis and Breton and Gorius were able to show by electron microscopy that the ferritin molecule enters the erythroblasts in lead poisoning but does not take part in the hemoglobin formation. Heilmeyer[615] classifies this anemia as a secondary sideroachrestic anemia, as this incorporated iron can be stained with Prussian blue reaction in the normoblasts as well as in the young peripheral red cells and the stippled cells. The failure to incorporate iron and the poor hemoglobin formation lead to a high serum iron level and to an increased urinary excretion of coproporphyrin III and of protoporphyrin. It may be that lead inactivates an enzyme which Goldberg[615] found in hemolysates of avian cells and which incorporates iron into the protoporphyrin ring. Other possible sites of interference with the hemoglobin synthesis are the formation of gamma-aminolevulinic acid and the protoporphyrin formation, according to Heilmeyer.[615]

In the peripheral blood the stippled cells keep company with polychromatic cells, and as shown by supravital staining there is an increased number of reticulocytes. The bone marrow shows a moderate erythroid hyperplasia, with basophilic stippling in late normoblasts.

It is believed that basophilic punctation is an effect of lead, but the presence of basophilic punctation alone does not prove the case. One must have history, clinical symptoms such as lead colic, the lead line on the gums, the finding of lead in the urine, etc.; all these serve to clinch the picture. P. Schmidt has well said that basophilic punctation is a "valuable aid before the appearance of other symptoms." There is a marked tendency today, with this disease entering into court circles, for litigants to attempt to convince juries, judges, and Workmen's Compensation Boards that the finding of basophilic punctation and polychromasia is proof positive of a case of lead poisoning or vice versa. It is fair to assume in these cases that in addition to the presence of basophilia the entire blood picture must be studied and weighed in the balance, as well as the clinical symptoms, before accurate conclusions may be drawn.

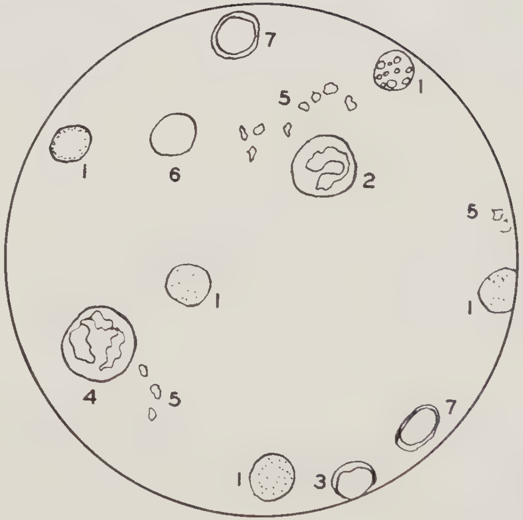
The **supravital methods of staining** are admirable for the demonstration of basophilic punctation.

The **thick drop method** of Schilling is the best method of demonstrating basophilic punctation and polychromasia in lead poisoning (pp. 1184ff). This method, originally introduced by Ross for the demonstration of malarial parasites, is now widely used in lead poisoning studies. An observation of the cen-



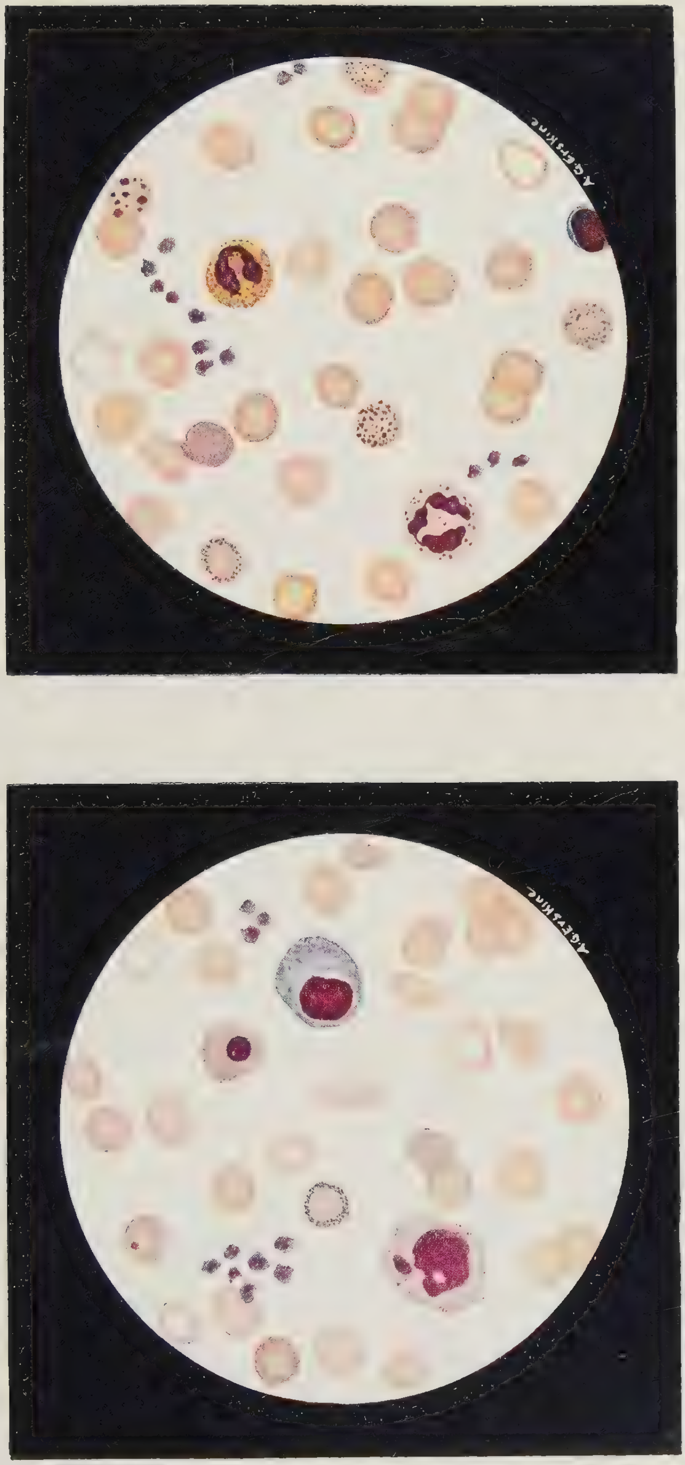
Hypochromic anemia
(Giemsa stain; ×950)

1. Blood platelet on an erythrocyte
2. Group of blood platelets
3. Polychromatic normoblast
4. Large lymphocyte with azurophilic stippling
5. Polychromatic erythrocyte
6. Basophilic punctated erythrocyte
7. Monocyte
8. Erythrocyte showing hypochromia



Lead poisoning
(Giemsa stain; ×950)

1. Basophilic punctated erythrocytes
2. Eosinophil
3. Lymphocyte
4. Segmented neutrophil
5. Blood platelets
6. Polychromatic erythrocyte
7. Erythrocyte showing marked hypochromia



Lead poisoning

Hypochromic anemia

Fig. 432

tral part of the drop will show the different degrees of basophilia present.

There has been some controversy as to just how to interpret this basophilic punctation in blood films and in thick drop preparations. Brueckner claims that no special significance may be attributed to basophilic punctation as compared with polychromasia and that the coarser forms of basophilic punctation are simply younger and perhaps more activated cell forms. Weight of opinion, however, seems to point to the fact that polychromasia and basophilic punctation are quite different. Polychromasia is found in marked degree in many conditions and under an endless variety of pathologic conditions, even in supposedly healthy persons who are probably under the influence of a slowly developing occult chronic illness. Basophilic punctation on the other hand is found only in very severe toxic anemias due to blood poisoning in various forms, protozoal disease, purpura, pernicious anemia, leukemia, and aplastic anemia. In other words, it is an entity due to a very definite and serious condition. When one can reasonably believe that no malaria exists, the first thought in finding a very pronounced state of basophilic punctation in the thick drop preparations or blood film would be lead poisoning or poisoning with benzol, arsenic, or with any one of the heavy metals. An objection has been raised to this conclusion by those who have studied the guinea pig experiments of Lehmann,[617-621] but one must remember that guinea pigs and man are quite different—that guinea pigs are quite sensitive and have been known to develop basophilic punctation from poor housing conditions. It must be mentioned here, however, that Konrich[622] and Lehmann[619-621] report basophilic punctation in cement workers. Jötten and Scharlau[623] failed to find basophilic punctation in workers in dusty occupations.

Whole blood lead levels of 0.09-0.3 mg.% are indicative of excessive lead absorption. Absorption is not synonymous with intoxication. (See Tables 252 and 253.)

Nonhematologic findings

Urine: Increased coproporphyrin and demonstration of intranuclear acid-fast inclusions in the sloughed renal tubular epithelium are noted.[616] In favor of excessive lead absorption is a lead level greater than 0.15 mg./L. Amounts greater than 0.2 mg./L. are indicative of excessive lead absorption. (See Tables 252 and 253.)

Summary

Lead poisoning is an acute disease characterized by symptoms involving 3 systems: the gastroenteric, hematopoietic, and nervous systems. Rarely the latter may appear to be exempt; the former 2 never are.

Acute episodes may recur and give a false appearance of chronicity. Sequelae also may persist for long periods. Signs of abnormal lead absorption are mistakenly considered to represent illness.

The cure of the disease depends on the cessation of hazardous exposure and the elimination of toxic quantities of lead from the body.

Kehoe[624] emphasizes this need for prophylaxis and decries the lack of proper preventive measures, as indicated by the great number of cases of lead intoxication that still exist. He particularly sounds a note of warning concerning the use or possible "misuse" of the newest agent in the treatment of lead intoxication—calcium disodium Versenate (ethyl-

Table 252. *Lead levels in health and in various industries**

	Normal lead levels	*Safe abnormal lead levels†*	*Hazardous abnormal lead levels‡*
Blood—mg./100 gm.	0.01-0.06 (average, 0.03)	0.01-0.07	0.07-0.4
Urine—mg./L.	0.01-0.08 (average, 0.03)	0.01-0.14	0.1-0.8
Stool—mg./gm. ash	0.005-0.09 (average, 0.03)	0.05-0.25	0.15-2.0
Spinal fluid—mg./100 ml.	0.003-0.001 (average, 0.001)	Less than 0.004	More than 0.004
Stippled red blood cells/50 oil immersion fields—12,500 cells	0-12 (average, 4)	Less than 16	16-500 (average, 75)
Average daily intake from all sources	0.05-2mg./day (average, 0.33)		

*Acknowledgment is made here to Dr. Robert A. Kehoe, Director of the Kettering Laboratory of Applied Physiology, University of Cincinnati, College of Medicine, for much of the information in this summary.

†Seen in certain industries with abdominal lead exposure at magnitudes not sufficiently great to cause clinical plumbism.

‡Cases of plumbism occur within this range. **Note:** Not all cases within this range have clinical plumbism.

Table 253. *Lead absorption in certain trades*

Subjects	*No. samples*	*Mean lead content*	
		Feces (mg./sample)	*Urine (mg./L.)*
Mexican Indian	29	0.11	0.022
Frenchman (no industrial exposure)	34	--	0.020
Normal American adult M.R.	31	0.38	0.021
Normal American adult H.D.	548	0.26	0.022
Germans (no industrial exposure)	13	--	0.027
Normal American adult E.B.	56	0.22	0.031
Medical students	71	0.24	0.038
Garage mechanics	81	0.51	0.047
Garage mechanics	71	0.54	0.050
Garage mechanics	140	0.47	0.051
Unexposed workmen	172	0.47	0.051
Workmen in paint mfg.	19	0.15	0.051
Workmen in insecticide mfg.	20	--	0.067
Workmen engaged in soldering	31	0.51	0.070
Workmen in tetraethyl lead mfg.	35	0.47	0.079
Workmen in tetraethyl lead mfg.	34	0.63	0.069
Firearms and bullet mfg. workers	74	0.48	0.097
Storage battery workers	21	1.73	0.128
White lead workers	40	2.70	0.176
White lead workers	26	4.00	0.191
Brass foundrymen	46	2.78	0.250
Storage battery workers	35	0.62	0.201
Storage battery workers	9	3.72	0.330
White lead workers	10	7.60	0.336

enediamine-tetraacetic acid, EDTA). Kehoe calls attention to the long list of substances which have been used in the past to mitigate the effects of excessive absorption of lead, such as milk, ascorbic acid, and laxatives and purgatives with lemonade fortified by acid. Although the derivatives of edathamil appear to have the property of combining with lead in the soft tissues and then being excreted in the urine, it has not been established that it is lifesaving or that it can be relied upon to relieve symptoms. For the relief of colic Vigliani[625] recommends that cortisone or corticotropin be administered along with drugs which either neutralize or eliminate lead from the body. Belknap and Perry[626] recommended the combination of intravenous calcium gluconate and edathamil calcium disodium for the treatment of lead colic.

*Symptoms of lead poisoning**
100% generalized weakness
90% weight loss
82% constipation
80% colic
77% anorexia
77% abdominal pain
70% arthralgia
57% lassitude
54% generalized aching
54% vomiting
50% nausea
43% insomnia
40% headache
40% metallic taste
30% abnormal dreams
27% excessive salivation
27% vertigo
10% impotence

*Physical signs in lead poisoning**
66% lead line
66% pyorrhea
60% dental decay
57% wrist extensor weakness
40% malnutrition
37% abdominal tenderness
37% infected tonsils
33% hyperactive biceps reflex
33% hyperactive abdominal reflexes
33% hyperactive cremasteric reflexes
30% tremor
27% hyperactive tendon reflexes of legs
27% pallor
20% sensory changes

Vital signs and clinical laboratory data
Temperature, 98.4° F.
Pulse, 80
Respiration, 22
Blood pressure, 132/79
RBC count, 4,270,000
WBC count, 7550
Hemoglobin, 11.4 gm.
Urine, occasional cast

Effects of other chemicals on the blood picture

In addition to lead the blood picture may be altered by other chemical substances. Acetanilid, acetophenetidin, and other aniline derivatives produce anemia. Stengel and White[627] reported a case of chronic acetanilid poisoning and severe anemia with red cells 2,000,000 and hemoglobin 35%. There were present a great many normoblasts with many variations in size and shape of the red cells.

Arsenic in small amounts over a long period of time causes a mild stimulation of hematopoiesis. When arsenic is ingested in toxic amounts, there is a reduction in the granular leukocytes, and actual leukopenia may occur.

Arsphenamine in exceptional cases has produced an effect upon the bone marrow very similar to that seen in aplastic anemia or

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*From a statistical analysis of 30 cases.

*From a statistical analysis of 30 cases.

Table 254. *Differential characteristics of the anemias*

	Beginning pernicious anemia	*Advanced pernicious anemia*	*Pernicious anemia under treatment*	*Iron-deficiency anemia*
Erythrocyte count	Slightly lowered	May be as low as 1,500,000 or lower	Let it go above normal as a rule in treatment	Around 4,000,000 (except in very severe cases)
Hemoglobin	Slightly lowered	Drops quite low but not as much proportionately as drop in red count	Rises under treatment	Very low, 50-60%
MCV* cμ	Slightly over 94	100 or higher	80-94	Less than 80
Blood vol.	Low	Below normal	Below normal	Very low
MCHC† %	Slightly above 30	Slightly above 30	Greater than 30	Less than 30
Leukocyte count	Slightly lower, if any change	Leukopenia (3000-4000)	Rises back to normal	Usually higher, around 10,000
Differential	Very little significant change; any change will be shift to the right	Leukopenia, neutropenia, right shift, hypersegmented neutrophils, lymphocytosis, monocytosis; in very serious cases may see myelocytes	Gradual return to normal	Lymphopenia; frequently a stab shift
Reticulocytes	Increase; go higher after vitamin B_{12} treatment	Depends upon amount of regeneration of erythrocytes	Begins to rise on 4-5th day and continues to rise until 10th day; drops to normal	Increased
Blood platelets	Begin to decrease	Low absolute	Begin to rise	Normal
Red cells in spread	Polychromasia; tendency of erythrocytes to enlarge	Anisocytosis with macrocytes, poikilocytosis, polychromasia, basophilic punctation, hyperchromia, macrocytes, megalocytes, and nucleated red cells; may find Howell-Jolly bodies, Cabot rings, marginal granules, erythrocytes, etc.	Return to normal; increase in polychromasia; disappearance of pathologic erythrocytic findings, as a good sign	Very marked hypochromia, poikilocytosis, anisocytosis, many microcytes, diffuse basophilia; normoblasts and microblasts may at times be present
Size of red cells	Majority are normal; tendency to enlarge	Large	Return to normal	Small
Pigment content red cells	Not significantly changed	Hyperchromic (increased)	Return to normal	Decreased
Special examinations needed	Blood vol. and vol. index; reticulocyte count; blood platelet count, van den Bergh; bone marrow puncture; gastric analysis	Reticulocyte count; routine complete blood examination; same as beginning pernicious anemia	Reticulocyte counts repeatedly; repeated red cell counts and hemoglobin	Complete blood picture
Bone marrow	Increase in megaloblasts	Great increase in megaloblasts; decrease in granulocytes	Return to normal, first showing increase in normoblasts	Not needed for diagnosis

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*Mean corpuscular volume.
†Mean corpuscular hemoglobin concentration.

Aplastic anemia	*Posthemorrhagic anemia (prolonged)*	*Sickle cell anemia*	*Chronic hemolytic anemia (idiopathic)*	*Lead poisoning*
Progressively lowering red count	Depends on severity of hemorrhage; rises as a good sign	Around 3,000,000	2,000,000-3,000,000, during attacks	2,500,000,-3,000,000
Progressively lowered; about same rate as red count	Depends on severity; usually quite low	Quite low; about same rate as red count	Drops proportionately to red cells	Very low; can go as low as 20%
80-94	Less than 80	80-94	80-94	Variable
Very low	Low	Low	Low	Low
Less than 30	Less than 30	Less than 30	Less than 30	Variable
Progressively lowered	High 10,000-15,000	High	High 25,000-30,000	Indefinite
Normal at beginning without young cells; later small lymphocytes up to 100%; never any immature white cells	Neutrophilia without shift	Shift; frequently regenerative	Very marked regenerative shift	Indefinite
Usually have none at all	Very rapid increase—depending on blood manufacture	Quite an increase	Very high	Extremely high
May completely disappear	Increase as a good sign	Very high	Very high	No significant change
Normocytic, normochromic; no change in erythrocytes noted	May see anisocytosis, marked polychromasia as a good sign; nucleated red cells in a severe hemorrhage; megaloblasts are serious	Anisocytosis, poikilocytosis, polychromasia, normocytic normochromic; nucleated red cells, basophilic punctation, Howell-Jolly bodies, Cabot ring bodies, endoglobular degeneration, target cells; sickling does not appear in film as a rule	Anisocytosis, poikilocytosis, marked polychromasia, many normoblasts, basophilic punctation, Cabot ring bodies, Howell-Jolly bodies, marginal granules, karyorrhexis, karyogenic metachromasia, megaloblasts, hypochromia, endoglobular degeneration, crescents	Marked polychromasia, basophilic punctation very coarse; may find other pathologic erythrocytes such as normoblasts, etc.
Normal	Normal, large, or may be small	Normal	Normal	Indefinite
Normal	Usually low	Normal	Normal	Indefinite
Bone marrow puncture; routine hematology serially	Reticulocyte count, serial red count, blood platelet count, hemoglobin test	Wet preparation; sometimes mix 1 drop patient's serum plus 1 drop patient's cells; sodium metabisulphite test and alkali denaturation test; electrophoretic studies	Reticulocyte count, resistance test, van den Bergh, icterus index, blood platelet, bone marrow puncture	Chemical analysis of urine and feces; reticulocyte count; thick drop
Either absence of nucleated reds or great decrease; only an occasional normoblast; may be fatty degeneration or infiltration of small lymphocytes; granulocytes disappear; may be plasma cells	Increase in normoblasts and eosinophils	Regenerative—packed with young red and white cells	Hyperplastic erythropoietic	In some cases basophilic stippling; not needed for diagnosis as rule

Continued on next page.

Table 254. *Differential characteristics of the anemias*—cont'd

	Beginning pernicious anemia	*Advanced pernicious anemia*	*Pernicious anemia under treatment*	*Iron-deficiency anemia*
Classification	Normocytic normochromic with macrocytes	Macrocytic normochromic	Return to normocytic normochromic	Microcytic hypochromic
Important points in diagnosis	Increase in size of red cells; increase in reticulocytes; response of patient to treatment with erythrocyte maturation factor (liver, vitamin B_{12})	Very low red count and hemoglobin and high color index; low white count, right shift and hypersegmentation of neutrophils; megalocytes and megaloblasts, hyperchromia; increase in reticulocytes following liver therapy; low blood platelets	Reticulocyte response to treatment; return of blood picture to normal	Tiny red cells with decreased pigment content; low color index

purpura hemorrhagica. This effect may be fatal.

Carbon monoxide produces secondary anemia. In acute poisoning, the hemoglobin changes to methemoglobin.

Nitrobenzene or shoe dye poisoning has been seen frequently. It is not at all certain that the union of the poison with hemoglobin produces methemoglobin so that the term pseudomethemoglobinemia has been devised by Peters and Van Slyke[628] for this condition. There are no changes in the morphology of the red cells.

Poisoning by benzol and related products

Benzol is a volatile inflammable fluid used in industry for cleaning printing rooms and engraving plates, in rubber cements, and in lacquers and varnishes and for many other purposes, being an excellent solvent for rubber, gums, fats, and resins.

Acute poisoning is often due to breathing excessively large amounts of benzol vapors. This may be rapidly fatal. Acute poisoning more often occurs as a result of an accident, such as the bursting of a pipe line, the overflow of a large tank, or workmen entering a tank for cleaning or repair work without using proper proctective measures.

Chronic poisoning differs from the acute type in that the benzol is inhaled in small quantities over a considerable period of time, the chief effects being observed in blood and blood-forming organs. It is a slow process, and the early stages and symptoms are not striking. Later, the symptoms increase, and if the condition is not recognized and exposure continues, serious poisoning may develop which in advanced cases may produce death.

Acute poisonings, for instance deaths in garages and poisoning in tanks, due to benzol (Marienfeld[629] and Zangger[630]) show no specific hematologic change. The subacute and the chronic benzol poisoning are suggestive of idiopathic blood diseases, and it is very difficult to distinguish them from thrombopenic diseases, such as Werlhof's purpura, agranulocytosis, aplastic anemia, panmyelophthisis, etc.

The first communication on this subject was made by Santesson[631] who reported 4 fatal cases of women workers in a velocipede factory; all 4 cases were among young women workers.

Santesson noted that with pure benzine experimentally no results were obtained, whereas with benzol (benzene), the same result was seen as in these 4 cases. In the autopsy material a study showed that severe endothelial fatty changes were responsible for the bleeding. Santesson believed that there was a central disturbance, which was first noted by Selling[632] whose clinical experimental work has been noted so often in the literature. Santesson reported 2 girls with chronic benzol poisoning, the first showing 640,000 erythrocytes and 8% hemoglobin. There was severe thrombocytopenia. In the second case there were 1,150,000 erythrocytes with 15% hemoglobin and no regenerative forms.

Benzol poisoning produces a depression of the entire bone marrow. It may lead to the various types of aplastic anemia.

HYPERSPLENISM

As has been seen in previous discussions, the spleen is always involved in hemolytic anemias, but if the splenic hemolytic activity becomes excessive and dominates the clinical picture, we speak of **hypersplenism.** Splenic

Aplastic anemia	*Posthemorrhagic anemia (prolonged)*	*Sickle cell anemia*	*Chronic hemolytic anemia (idiopathic)*	*Lead poisoning*
Normocytic normochromic	Microcytic hypochromic; normocytic normochromic	Normocytic normochromic	Normocytic normochromic	Indefinite
Entire blood and bone marrow pictures taken serially, giving picture of true aplasia	Rising red count and hemoglobin, increase in reticulocytes, high blood platelet count as good signs	75-100% of red cells sickle-shaped in wet preparation; electrophoresis shows hemoglobin-S; fetal hemoglobin increased	Entire blood picture and special tests needed; search for isoimmune and autoimmune bodies	History of exposure to lead, clinical symptoms, chemical analysis of urine and feces, +++ and ++++ polychromasia in thick drops; basophilic punctation

hemolytic anemia can be divided into 2 groups—primary and secondary.

Primary splenic hemolytic anemia

The etiology of primary splenic hemolytic anemia is unknown. It represents an exacerbation of the normal splenic function which retards the flow of red blood cells, changes their surface tension, and destroys them (see discussion of the function of the spleen). The dysfunction may find expression in increased phagocytosis or in increased hemolysis. The production of a humeral substance acting upon the bone marrow, either affecting all hematopoietic elements or only one or the other, and the release of the hypothetical toxic splenic substance may both lead to the production of a nonhemolytic splenic anemia (**dysplenism of Hittmair**).

Secondary splenic hemolytic anemia

Secondary splenic hemolytic anemia may accompany splenomegaly, irrespective of its etiology. It may thus appear as a complicating factor in congestive splenomegaly (e.g., cirrhosis of the liver), malaria, lymphomas, infectious mononucleosis, etc.

SPURIOUS ANEMIA

Before leaving the subject of anemia, it should be pointed out that it is at times difficult, on the basis of borderline laboratory data, to establish the diagnosis of an anemia. Wilson and Boyle[633] and Wiseman[634] have pointed out that a relative anemia may be caused by an increase in plasma volume. The red cell count and the hemoglobin level give the number of red blood cells and the amount of hemoglobin per unit volume. Because of its inherent error due to the trapped plasma, the venous hematocrit is usually 10-15% lower than the body hematocrit. Hemodilution may thus be interpreted as anemia, whereas in actual fact the circulating red cell mass and the circulating hemoglobin are normal. In these cases, total blood volume, plasma volume, or the erythrocytic mass should be determined by isotope methods.

DIFFERENTIAL CHARACTERISTICS OF THE ANEMIAS

Table 254 was developed at the Gradwohl School of Laboratory Technique to aid in teaching the laboratory findings in some of the anemias. It is not intended to be a complete review of the various anemic conditions, but simply a handy reference.

Chapter 65

Affections of the white blood cells

JOHN D. BAUER

An increased number of leukocytes is called **leukocytosis** and a decreased number, **leukopenia.**

LEUKOCYTOSIS

Variations may occur in the blood of the same individual even in health from day to day or at different hours of the same day. These variations may be due to an increase in any one of the elements, i.e., granulocytes, lymphocytes, or monocytes. In other words, there may be a granulocytic leukocytosis, lymphocytic leukocytosis, or monocytic leukocytosis. The purpose of the differential count is to tell what type of leukocytosis is at hand, whether it is granulocytic, lymphocytic, or monocytic. As a rule leukocytosis is usually granulocytic or neutrophilic. When we speak of neutrophilia we mean a neutrophilic leukocytosis.

Physiologic neutrophilic leukocytosis

Some authorities have spoken of digestive leukocytosis, i.e., an increase in leukocytes during digestion, but this seems to be a misconception. There seems to be a variation in the total white cell count during the day, usually a maximum of 9000 to 10,000 in the afternoon and 5000 to 8000 in the morning. These variations occur quite independently of meals. Leukocytosis has been noted in pregnancy and during menstruation and also as a result of muscular exercise. In this category we must also mention the physiologic leukocytosis of the newborn infant, of emotional disturbances (excitement, fear), and of exposure to sunlight (ultraviolet rays).

Pathologic neutrophilic leukocytosis: It occurs in the following conditions: (1) bacterial infections—infections caused by the pyogenic bacteria (pneumococci, streptococci, staphylococci, etc.), (2) metabolic disturbances—diabetic coma, uremic coma, and acidosis, (3) blood dyscrasias—hemorrhage, hemolysis, myeloproliferative disorders, and granulocytic leukemia, (4) drug administration—digitalis, mercury, phenacetin, and ACTH, (5) tissue breakdown—myocardial infarct, burns, gangrene, and tumors, and (6) allergy, vaccines, cold, and heat.

Shift of Schilling

It is generally believed that the following order of cell development occurs in the myelocytic series: (1) the myeloblast of Naegeli, (2) the promyelocyte of Pappenheim, (3) the myelocyte of Ehrlich, (4) the juvenile of Schilling, (5) the stab or band form of Schilling, and (6) the segmented cell. The last 3 are what were formerly termed **polymorphonuclear cells.** We must assume that the bone marrow is irritable or responsive or highly sensitive to an acute degree to chemotactic, humoral, and vegetative-nervous insults, that it is the primary carrier of cellular defensive forces against toxic substances in the body, especially proteogenous antigens. It was Arneth who first called attention to the left shift of neutrophilic leukocytes as a very important development of histologic changes in connection with clinical diagnosis and prognosis. All the credit must be given to him for calling attention to these nuclear changes. His work, however, is not strictly speaking a practical procedure, nor do the other types of cells which he has described in his book *(Qualitative Blutlehre)* conform to the original idea of a shift of the neutrophils. The total scheme comprises an observation of more than 80 cell species. The Schilling count divides cells as follows: basophils, eosinophils, myelocytes, juvenile cells, stab or rod cells, segmented cells, lymphocytes, and monocytes. The normal quantities are basophils, 0-1; eosinophils, 2-4; myelocytes, 0; juvenile cells, 0-1; stab cells, 3-5; segmented cells, 58-66; lymphocytes, 21-30; monocytes, 4-8. The neutrophils are myelocytes, juvenile, stab, and segmented cells, or a total of 67. He has proved that in the presence of infection there is a definite change in the proportion of these cells. In the beginning, due to the toxic impression upon the bone marrow, there is an interruption in the development from stab to segmented cells, with a consequent increase in stab cells.

To set forth the cells according to the Schilling method, construct a so-called hemogram as shown in Table 255.

Now draw a double line between the segmented and the stab cells. If the neutrophilic cells to the left of this line increase, it is called a left shift. If the neutrophilic cells to the right increase, together with a decrease or absence of these cells on the left of the line, it is called a right shift. The true right shift shows numerous hypersegmented (6 or more lobes) neutrophils.

A simple **degenerative blood picture** is a simple left shift in which the stab cells increase in number. With a continued infection and with the defensive forces of the body acting intensively under infection, this shift continues perhaps with the stab cells remaining stationary or increasing, but with the appearance of juvenile cells in the peripheral blood. At this time the appearance of juvenile cells indicates an irritation of bone marrow with regeneration. This is called a **regenerative blood picture.** If the infection continues and the patient's resistance declines, the shift advances farther to the left. If improvement ensues, the shift declines and recedes to the right. A **leukemoid shift** is a shift in which the blood picture resembles leukemia, i.e., a marked increase in myelocytes, juvenile cells, stab cells, and segmented neutrophilic cells. In this type of shift, there are no eosinophils or basophils, and the number of lymphocytes and monocytes is very low; sometimes there are none. This is a serious blood picture.

Attention is also to be called to the change in number of lymphocytes and monocytes during this infectious period. With a beginning shift to the left there is a coincident decline in lymphocytes. This decline continues until recuperation begins. With recuperation and retrogression of the shift toward the right, the lymphocytes increase. Manifestly a decline in lymphocytes indicates beginning infection, and increase in lymphocytes indicates resistance against infection. The neutrophilic regenerative shift implies a battle phase. Decline in lymphocytes indicates that the fortunes of war are with the invading organism, but with receding neutrophilia, a rising number of lymphocytes means a phase of cure. At some stage in the infectious period when resistance overcomes invasion, the monocytes show a sudden rise. This occurs often even before the shift is materially changed to the right and is termed the **phase of defense.** In the beginning of infections the eosinophils decline and often totally disappear and reappear only when the shift turns about from left to right. This is termed the **dawn of convalescence.** There is found, therefore, as a favorable prognosis, a return of eosinophils, a shift of neutrophils, changing from the left to the right, an increase in lymphocytes, and a marked increase in monocytes.

In chronic infectious conditions, a persistent high shift with low counts of eosinophils and high counts of monocytes shows a chronic simultaneous continuance of a phase of neutrophilic struggle and of monocytic defense. Thus the Schilling hemogram presents a kaleidoscopic view of infection in the blood.

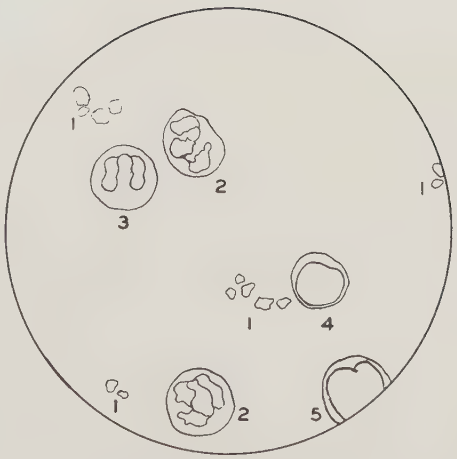
We are enabled to note infection, to note susceptibility, and to note resistance. We are also able to note, very clearly, an increase in the severity of the infection and can predict, with certainty, impending death. It is in impending death that we see the flooding or liberation in which the bone marrow has finally been overcome, and the peripheral blood shows almost intact cells ordinarily found only in the bone marrow.

Broadly speaking, the Schilling hemogram

Text continued on p. 1318.

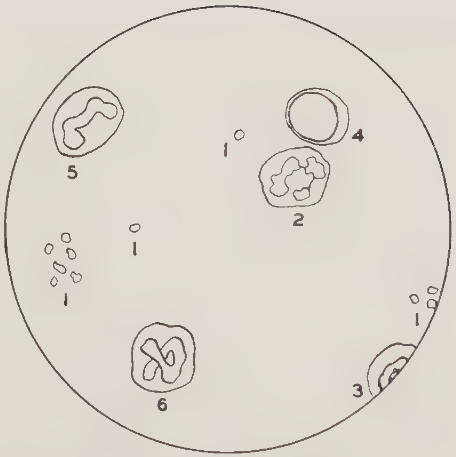
Table 255

			Total neutrophils					
	Basophils	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*
Normal %	(0-1)	(2-4)	(0)	(0-1)	(3-5)	(58-66)	(21-30)	(4-8)



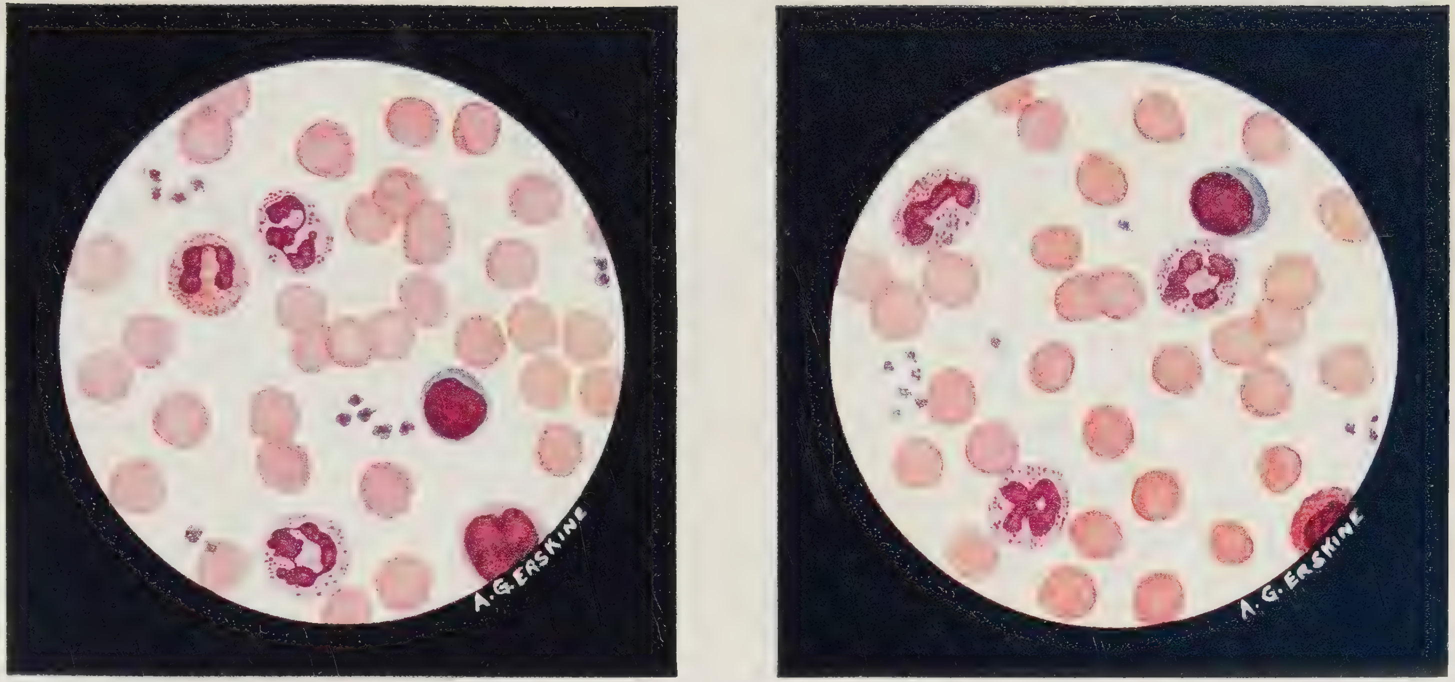
Normal blood picture
(Giemsa stain; ×950)

1. Blood platelets
2. Segmented neutrophil
3. Eosinophil
4. Lymphocyte
5. Monocyte



Mild grade infection
(Giemsa stain; ×950)

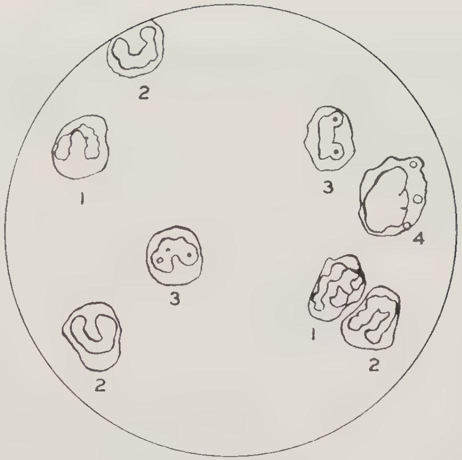
1. Blood platelets
2. Segmented neutrophil
3. Eosinophil
4. Lymphocyte
5. Stab cell
6. Stab cell with twisted nucleus


A.G.ERSKINE
A.G.ERSKINE

Normal blood picture

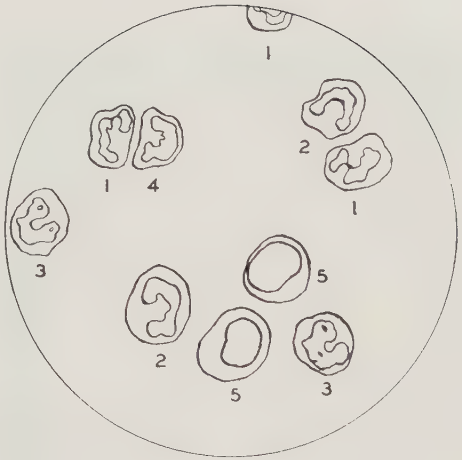
Mild grade infection

Fig. 433



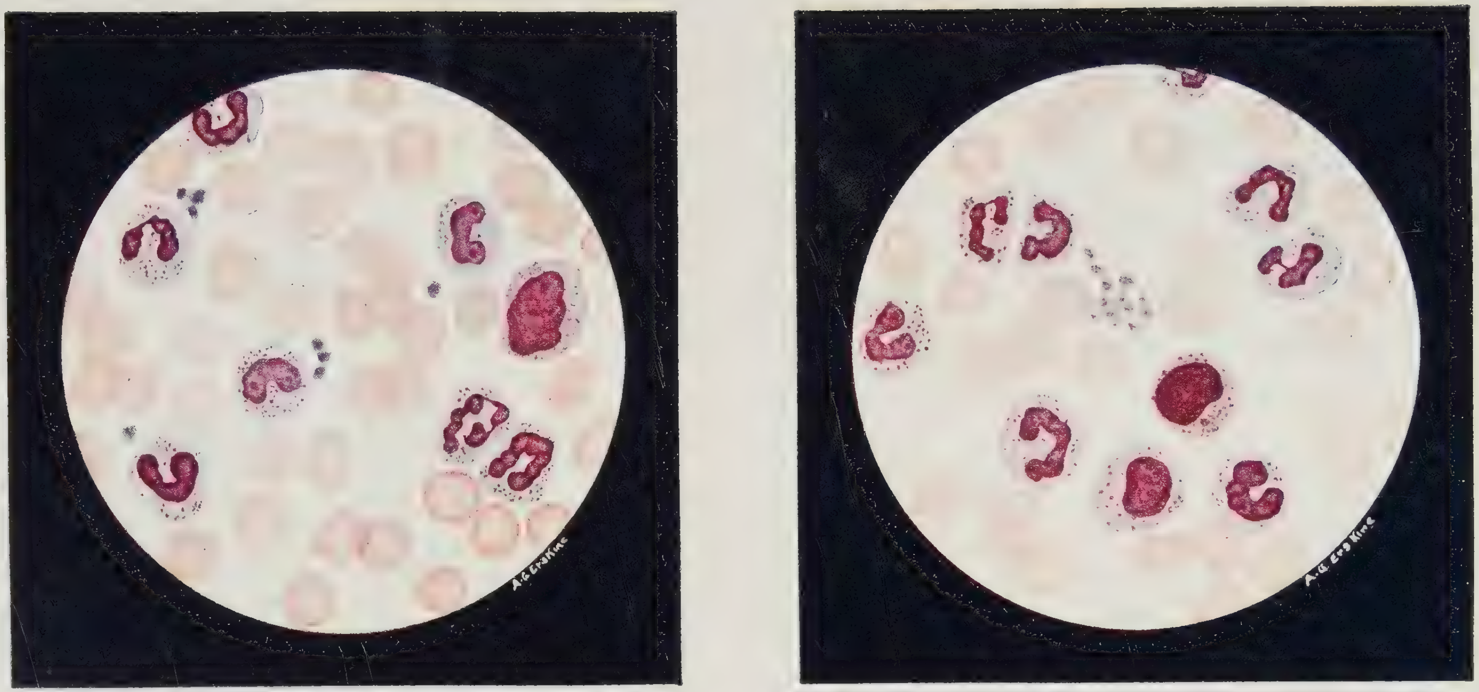
Severe grade infection
(Giemsa stain; ×950)

1. Segmented neutrophils
2. Stab cells
3. Juvenile cells
4. Monocyte



Fatal grade infection
(Giemsa stain; ×950)

1. Segmented neutrophils
2. Stab cells
3. Juvenile cells
4. Degenerated stab cell
5. Neutrophilic myelocytes


A. G. Erskine
A. G. Erskine

Severe grade infection

Fatal grade infection

Fig. 434

shows a biologic reaction to disease but not necessarily a specific picture for each infection. It is true that there are certain conditions of infection which give rather specific blood pictures, but it does not necessarily follow that a diagnosis of a specific infection can be made entirely by the blood picture. Certain conclusions can be drawn very accurately in many infections from the blood picture, for instance, in pneumonia, which is characterized by a hyperleukocytosis and a neutrophilia with regenerative shift. The blood findings in tuberculosis are highly interesting. With activity there is a marked shift with a decline in lymphocytes and with resistance a recession of neutrophils to the right and a marked increase in lymphocytes.

In typhoid fever there is a very interesting degenerative left shift and leukopenia. Particularly interesting is the aneosinophilia in typhoid fever. In typhus, on the contrary, we find what Schilling is pleased to term the **gay blood picture** which shows a marked increase in stab cells and quite a few juvenile cells, in other words, a regenerative blood picture, together with irritation forms and plasma cells and an increase in monocytes. This gay blood picture is also seen in measles and German measles but is less marked. It is to be noted that monocytes with an increased white cell count are conspicuous in variola but less marked in varicella, scarlet fever, and mumps.

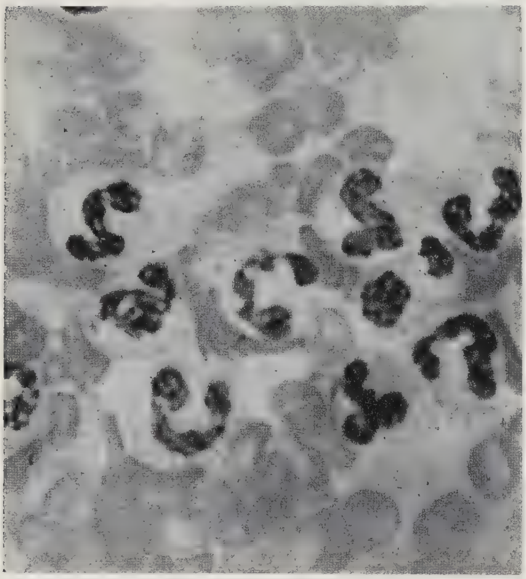


Fig. 435. Tuberculosis, blood film, showing increase in stab cells and segmented cells. (×950.)

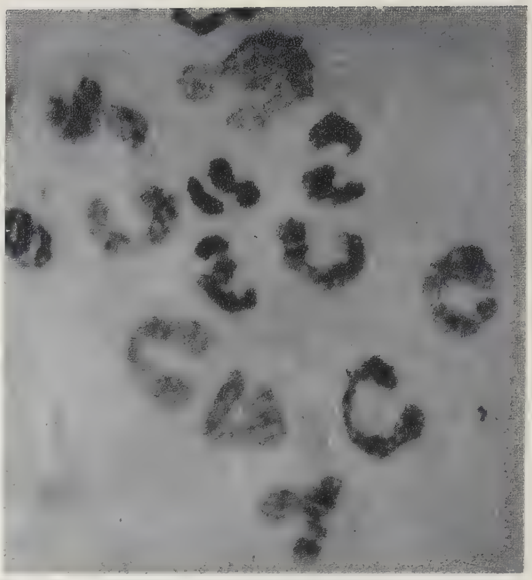


Fig. 436. Acute infection, blood film, showing increase in juvenile and stab cells. (×950.)

It goes without saying that the Schilling differential count presupposes a knowledge of the exact appearance of the different types of blood cells. It is exceedingly important to recognize the difference between a juvenile, a stab, and a segmented neutrophil. It is equally important not to confuse the lymphocytes with the monocytes.

The **nuclear shift index** (Schilling) is used particularly when the number of neutrophils in the hemogram is low. The exclusive use of the index obscures the interpretation. The nuclear shift index is the relation of pathologic neutrophils (myelocytes + juvenile cells + stab cells) to segmented forms. Normally the index is 4:64 or 1:16. Thus in a blood picture with no myelocytes, no juvenile cells, 2 stab cells, and only 18 segmented forms, the shift index is 1:9, which is a left shift.

Absolute counts of leukocytes

Absolute counts of each type of leukocyte are seldom made, but at times they are valuable to determine whether there is overactivity or underactivity of certain hematopoietic systems when the differential count alone does not show this clearly. To obtain the absolute count of each type of white cell, multiply the patient's white cell count by the % of each cell as represented in the differential count.

Normally the average white count is 7500 leukocytes. The normal differential count of Schilling is as shown in Table 256.

Table 256

Basophils	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*
0-1	2-4	0	0-1	3-5	58-66	21-30	4-8

Table 257

Basophils	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*
0 to 75	150 to 300	0	0 to 75	225 to 375	4350 to 4950	1575 to 2250	300 to 600

Table 258

	Basophils	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*
White cell count								
2400	–	–	–	–	–	35	60	5
Absolute cell count	–	–	–	–	–	840	1440	120

The absolute count in a normal individual, roughly, would be as shown in Table 257.

In Table 258 the absolute counts are given below the differential count.

At first glance it appears from the differential count that there has been alteration in function of the lymphopoietic system which resulted in an increase of lymphocytes. From the absolute count this is clearly seen as only a relative change. The absolute number is slightly below the normal limits.

Eosinophilic leukocytosis

An increase in the number of eosinophils or **eosinophilic leukocytosis** occurs in general in allergic states, such as certain skin diseases, asthma, and anaphylactic shock, and in infestations with animal parasites such as the hookworm and *Trichinella spiralis.* We have seen as many as 90% eosinophils in this latter condition accompanied by a marked increase in the total number of white cells. Other causes of eosinophilia are blood diseases—myelogenous leukemia, familial eosinophilia, Hodgkin's disease, polycythemia—and at times subacute infections such as tuberculosis or healing pneumonia.

In a general sense the increase in the total number of leukocytes constitutes a normal reaction on the part of the blood. In disease, however, such as leukemia, associated with an increased number of leukocytes, this increase is a primary feature and not a reaction of defense in any sense.

Basophilic leukocytosis

Myelogenous leukemia
Polycythemia vera

Lymphocytosis

- Viral infections
  - Infectious lymphocytosis
  - Mumps
  - Pertussis
  - Infectious mononucleosis
  - Infectious hepatitis
  - Viral pneumonia
  - Measles
- Bacterial infections
  - Tuberculosis
  - Brucellosis
  - Syphilis
  - Healing infections
- Blood diseases
  - Lymphatic leukemia
  - Felty's syndrome
  - Banti's syndrome
  - Infectious lymphocytosis
  - Infectious mononucleosis
- Hormonal diseases
  - Hypothyroidism
  - Hypoadrenalism

Monocytosis

- Viral infections
  - Chickenpox
  - Mumps
- Bacterial infections
  - Subacute endocarditis
  - Tuberculosis
  - Weil's disease
- Miscellaneous causes
  - Malignant tumors
  - Cirrhosis of the liver
  - Banti's disease
  - Monocytic leukemia

SPECIAL INTERPRETATION OF HEMOGRAM

In acute infections:

1. All blood pictures with beginning regenerative shifts and moderately high white cell counts are considered more favorable than those with degenerative shifts; nevertheless, rapidly increasing or very marked regenerative changes must be cautiously interpreted, especially if the white count decreases.

2. Marked signs of degeneration with decreasing white cell counts indicate a continuation of the infection.

3. Hyperregenerative and atypical hyperplastic blood pictures are serious symptoms that demand rapid removal of the cause; otherwise irreparable damage to hematopoiesis takes place and death will follow.

4. Constant aregenerative or aplastic blood pictures with low white cell counts are serious symptoms.

5. Approximately normal blood pictures and percentages must be seriously interpreted until sufficient time has elapsed to convince the **experienced** observer that the probable impending grave condition will not develop. The type of the hemogram, **determined by experience,** must always form the basis of a general estimation in any given case.

6. Marked decrease of eosinophils accompanied by rising leukocytosis indicates an **aggravated** condition.

7. Total disappearance of eosinophils (aneosinophilia*) with simultaneous marked lymphocytopenia is to be regarded as an **unfavorable** condition.

8. Constant presence of eosinophils, especially if this parallels hyperleukocytoses, means a **favorable** situation.

9. Reappearance of eosinophils (after absence from the thick drop preparation) or increase in eosinophils, with receding neutrophilia, with transition from lymphocytopenia to lymphocytosis, means a **very favorable** condition.

10. Moderately high white cell count and moderate differential change without or with slight shift and with eosinophils means a **favorable** condition.

11. The same picture in chronic conditions as an indication of a torpid cure shows a **doubtful** result.

12. Moderate or high white cell count with marked left shift, few eosinophils, and slight decline in lymphocytes indicates a condition not yet to be regarded as unfavorable.

13. Very high white cell count with marked left shift, with very young cells and marked neutrophilia, decrease of lymphocytes, and aneosinophilia suggests a **doubtful** or **unfavorable** condition, especially if these findings become more pronounced.

14. Decreasing low white cell count or leukopenia with very high neutrophilia and beginning shift to the left, lymphocytopenia, and aneosinophilia indicates a fatal prognosis.

15. Varying high white cell counts with constant hyperregenerative leukemoid or even irregular blood pictures mean a very **unfavorable** outcome.

16. Subnormal white cell counts with degenerative shift of neutrophils, lymphocytosis, monocytosis, and presence of eosinophils per se are **not unfavorable.**

17. Very low white cell counts with severe degenerative shift, neutropenia, and aneosinophilia, with only relative lymphocytosis and monocytosis, indicate an **unfavorable** state.

18. The retrogression of an existing shift (regenerative or degenerative) and of the neutrophilia or neutropenia with approach to the normal count and with reappearance of the eosinophils is to be regarded as **always** favorable.

19. On the contrary a blood picture showing a marked left shift deviating extensively from the normal count and accompanied by a decrease in or total disappearance of eosinophils is **unfavorable,** and the more extensive the deviation from the normal, the more unfavorable is the picture.

20. Marked decrease of lymphocytes in infections in which there have been hyperleukocytoses of long duration shows a **complication.**

21. Sudden decrease of lymphocytes with increasing neutrophilia and shift often with recurrent decreasing total white cell count (fall of lymphocytes) is a **very serious condition.**

22. Parallelism between lymphocytosis and hyperleukocytosis is a **favorable symptom.**

23. Transition from neutrophilia to neutropenia with receding shift, with increasing lymphocytosis, and eosinophilia is a **very favorable condition.**

24. Numerous younger or irritation lymphocytes with normal or abnormal white cell counts indicate active processes.

25. In acute infections with hyperleukocytoses, monocytosis is regarded as a **favorable** symptom, receding only in more severe affections.

26. In chronic infections, monocytosis indicates activity.

In anemias:

1. Considerable polychromasia or normoblasts without degenerative elements in the film with increasing red cell count and color index near 1.0 indicate a **favorable** condition.

2. Very marked regeneration (numerous normoblasts and erythroblasts, considerable polychromasia), and a low red cell count is to be considered a **doubtful** picture.

3. A low red cell count, decreasing hemoglobin, and decreasing color index without much polychromasia and signs of degeneration (basophilic stippling, isolated normoblasts, nuclear débris, special forms) means an **unfavorable** picture.

4. A low red cell count, leukopenia, lymphocytosis, megalocytosis, and color index above 1.0 (megalocytic or pernicious anemia blood picture) mean an **unfavorable** picture.

5. Constant low red and white cell counts without regeneration and followed by degeneration or aplasia is distinctly a very **unfavorable** condition.

BLOOD PICTURE IN VARIOUS INFECTIOUS DISEASES

Allergic states: The majority of hematologists regard eosinophilia, together with a leukopenia, as a very marked sign of allergy.

Amebiasis: In amebic dysentery there is a secondary anemia, and in severe chronic processes the erythrocyte count may drop to as

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*No eosinophils in the thick drop preparation.

Table 259. *Hemograms to illustrate interpretations given in acute infections*

Example No.	*Leukocytes*	*Basophils*	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*	*Remarks*
1a	12,300	–	–	–	4	16	51	25	4	Patient 1 (more favorable than patient 2)
	3,800	–	–	–	–	32	38	24	6	Patient 2
1b	12,300	–	–	–	4	16	51	25	4	Patient 1—Monday
	8,000	–	–	–	8	22	52	12	6	Tuesday: change for worse
2	7,800	–	1	–	–	10	61	25	3	Consecutive counts on same patient; infection progressing (unfavorable but not necessarily fatal)
	6,000	–	(+)	–	–	15	60	20	5	
	5,000	–	–	–	–	25	57	12	6	
3	23,000	–	(–)*	5	32½	28½	20½	8	4½	Hyperregenerative shift; very unfavorable
4	2,500	–	–	–	–	–	18	75	7	Aregenerative shift; very unfavorable
6	8,600	½	3	–	–	6	58½	26½	5½	Consecutive counts on same patient; advancing infection
	9,800	–	½	–	½	9	66	15	9	
	14,300	–	(–)	–	2½	14½	66	9	8	
7	16,000	–	(–)	½	15	30	41½	12½	½	Absence of eosinophils; unfavorable
8 and 9	8,000	–	–	–	19	17	31	19	14	Nov. 23
	7,800	–	(+)	–	11	26	27	21	15	Nov. 24
	7,600	–	1	–	–	1	56	29	13	Dec. 14
	10,000	–	15	–	–	–	57	22	6	Jan. 5
10	11,900	–	4	–	–	10	65	14	7	Moderate infection; favorable
12	15,000	–	½	–	½	21	58	17	3	Infection not yet regarded as unfavorable
13	23,000	–	(–)	5	33½	18½	20½	8	14½	Doubtful or unfavorable
14	10,000	–	(–)	–	2	8	81	7	2	Same patient
	4,800	–	(–)	–	3	10	83	1	3	Fatal picture
15	28,000	–	(–)	2	26	19	38	8	7	Unfavorable
16	4,000	–	(–)	–	–	20	30	40	10	If typhoid fever, picture is typical and not unfavorable; if erysipelas or other acute infection, picture is very unfavorable (serious)
17	3,900	–	(–)	–	–	34	23	35	8	Very unfavorable in acute infections
18	13,800	–	(+)	–	2	19	50	21	8	Consecutive counts on same patient; favorable outcome
	12,000	–	1½	–	1	11	53½	23½	9½	
	10,000	–	1	–	–	4	59	25	11	
	10,000	–	5	–	–	6	59	20	10	
	8,000	–	2	–	–	2	67	20	10	
19	Very high	–	4	–	6	21	46	14	9	Consecutive counts on the same patient; unfavorable outcome
	Slightly lower	–	3½	–	9½	27	42	12	6	
	Slightly lower	½	½	–	10½	20½	39	21	8	
	Slightly lower	–	(–)	–	24	33	34	7	2	
	Extremely low	–	(–)	1	22	24	33	13	7	
20	18,000	–	1	–	2	20	50	20	7	Complication
	18,000	–	1	–	2	28	54	8	7	
	19,050	–	–	1	10	30	50	6	3	

*(–) no eosinophils in the thick drop preparation.

Table 260

Total white cell count	*Basophils*	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*
First day, 10,850	0	12	0	0	5	48	33	2
Second day, 10,450	0	12	0	0	5	55	23	5

low as 2,000,000, with a still greater reduction in hemoglobin. There is hyperleukocytosis and a marked left shift. Eosinophilia is insisted upon by some and denied by others. It is a general belief of hematologists that the presence of eosinophilia indicates infestation with intestinal worms as a complication.

When amebic abscess of the liver supervenes, there is no special increase in leukocytes.

Appendicitis: The hemogram is of some assistance in elaborating the diagnosis of appendicitis; *one is in no way able to predict from the hemogram alone the exact degree of pathology which will be found at operation or after microscopic study.* Some clinical pathologists and surgeons attempt to draw conclusions of too refined a character from the hemogram in the presence of a disturbance in the lower right quadrant. We wish to emphasize that the study conducted by Gradwohl,* together with other experiences, would cause us to hesitate to draw any conclusion other than the presence of inflammation of the appendix from any given hemogram. We do, however, acknowledge that a falling total count and an increased left shift is a prime indicator of a probable rupture, with severe peritonitis and possibly a fatal outcome. It can be noted that, first, there may be an irregular fever in all types of appendix inflammation; second, there is a difference in the degree of symptoms; third, there is very little difference in the height of the white cell count and in the left shift in most cases whether they are so-called acute or ruptured appendicitis or chronic appendicitis with acute exacerbation. The distinction, if any, may possibly be a more pronounced left shift, but this is not a sufficient basis upon which to make an absolute differentiation. The blood picture in the chronic cases with intermittent flare-ups shows leukocytosis, together with a left shift, although this leukocytosis in a general way may not be as high as that commonly found in the acute conditions. Attention must also be called to the fact that in the case of fecal impaction without much pathologic disease in the appendix wall, there is a mild but definite left shift.

Bubonic plague: There is neutrophilic leukocytosis, around 40,000. Platelets are increased. There is a very marked left shift.

Coccidioidal granuloma: There is moderate leukocytosis, usually around 15,000. Eosinophils are sometimes above normal. Riseman and Ahlfeldt[635] reported a case with 22,000 white cells and 12% eosinophils. There is a secondary anemia.

Diphtheria: There is characteristic leukocytosis in diphtheria. With the injection of diphtheria antitoxin, there is a rapid decline in the number of leukocytes. Schlessinger[636] investigated leukocytosis in experimental diphtheria in animals. After injection of diphtheria bacilli, he noted leukopenia together with a decrease in lymphocytes, and then came an increase in leukocytes. Heim[637] claims that leukocytosis runs parallel with the intensity of the infection. Others note an absolute and relative increase in neutrophils, together with a decline in lymphocytes in the beginning of the disease.

Encephalitis lethargica: This is characterized by a slight increase of leukocytes, with a moderate degree of anemia. There is a slight left shift. Some authors have reported leukopenia and also eosinophilia during the convalescent period. The type of encephalitis that occurred in St. Louis in 1933 showed slight leukocytosis and a left shift.

Erysipelas: There is a hyperleukocytosis with a marked regenerative shift to the left. Total neutrophils may attain a figure of 90.

Hansen's disease: The predominant feature of the blood in Hansen's disease is an anemia. Leukocytes are not increased, and eosinophilia is not common.

Influenza: Influenza is characterized by leukopenia with a left shift. A typical hemogram is shown in Table 261.

Measles: In measles there are no characteristic changes in the hemoglobin content value or the count of the red cells. At the beginning of the disease there is neutrophilic

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*Gradwohl, R. B. H.: J. Missouri M. A. **30:**123, 1933.

leukocytosis followed rapidly by neutrophilic leukopenia. The leukocytes appear during incubation, and the leukopenia is seen at the time of eruption. Leukopenia is almost continuous, the lowest count recorded being 2400 during the height of the disease. Usbeck found leukopenia in the beginning inconstant. Hecker found, during the incubation period at the time of the appearance of the Koplik spots, leukopenia as well as relative and absolute lymphocytopenia with a left shift, and a decrease in eosinophils. In the second half of the period of incubation, there is an increase in lymphocytes and a decrease in neutrophils.

In the exanthematous period there is leukopenia and an absolute and relative decrease or absence of eosinophils. This typical blood picture was found 12 times in 41 cases examined. In recrudescence there are leukocytosis and relative lymphocytosis and eosinophilia. There is not a left shift.

With complications, such as pneumonia, otitis, etc., we may find leukocytosis, neutrophilia, and a left shift. The blood platelet count at the beginning of the exanthematous period is decreased to 100,000.

During the eruption there is a marked left shift with predominance of young forms. There are plasma cells and the usual gay blood picture of Schilling.

Paratyphoid A and B infections: Paratyphoid A and B show a reduction, possibly to ½ million, of red cells with a lowered hemoglobin content. The leukopenia is not as pronounced as it is in typhoid fever. The left shift is about the same.

Parotitis epidemica (mumps): Lehndorf[638] reported the blood pictures of 14 cases of mumps in adults. Leukocytes were normal. Lymphocytes were increased 30-65%. Two % plasma cells were found. Neutrophils were decreased, as were eosinophils.

Wiese's[639] findings in 115 cases of mumps from the ages of 5 to 15 yr. are given in Table 262 (no changes in the hemoglobin and red cell count).

There are a marked left shift and lymphocytosis together with leukopenia in the beginning of the disease.

Pneumonia: In pneumonia there is marked hyperleukocytosis, together with a very severe left shift. The total count may exceed 35,000, especially in children, when it may be 50,000. This is marked neutrophilic leukocytosis with a left shift. Occasionally myelocytes may be found during the height of the disease. The leukocytosis occurs very soon after the beginning chill and remains until the crisis occurs. With the crisis, there is a return of eosinophils and an appearance of monocytes—the so-called dawn of convalescence. One is accustomed to think of severe hyperleukocytosis in pneumonia as a favorable symptom and the absence of leukocytosis as a very unfavorable symptom. If leukopenia is found in bacterial pneumonia, the prognosis is very grave. There is very little change in the red cell picture and only a very slight reduction in the erythrocytes and hemoglobin. Blood platelets are increased, and the sedimentation rate is markedly heightened.

Pertussis: In pertussis there is leukocytosis, sometimes as high as 40,000 or higher. The

Table 261

White cell count	*Basophils*	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*
4000	0	0	0	0	15	58	23	4

Table 262. *Hematologic findings in 115 cases of mumps*

Day of disease	*Basophils (%)*	*Eosinophils (%)*	*Myelocytes (%)*	*Juvenile cells (%)*	*Stab cells (%)*	*Segmented cells (%)*	*Neutrophils (%)*	*Lymphocytes (%)*	*Monocytes (%)*	*Neutrophils*	*Lymphocytes*	*Total leukocytes*
1	–	–	–	–	–	–	–	–	–	–	–	–
2	1	5	–	2	9	21	32	47	15	1760	2585	5,500
3	2	5	–	1	6	14	21	59	13	1144	3216	5,450
4	1	3	–	–	1	11	12	68	10	972	5508	8,100
5	1	12	–	1	4	17	22	57	8	1771	4589	8,050
6	–	6	–	1	1	27	29	55	10	2088	3960	7,200
7	1	7	–	2	2	36	40	48	4	4080	4896	10,200
10	–	–	–	–	–	–	–	–	–	–	–	11,600
11	1	7	–	2	2	40	45	44	3	4896	6820	13,000

younger the child, the more outstanding is the leukocytosis. This leukocytosis is practically a lymphocytosis. In 24 cases in the catarrhal stage, there was an average of 20,000 white cells, of which 32% were neutrophils and 66% were lymphocytes. The highest leukocytic and lymphocytic figures are found during the paroxysm of the disease. Complications by streptococcus, pneumonia, or staphylococcus do not seem to influence the count. In a typical pertussis case Thiemann found 42,000 leukocytes, with 76% lymphocytes and 19% neutrophils.

There is a left shift of the neutrophils in either the catarrhal or paroxysmal stages. Count of the eosinophils is not changed. The monocyte count is normal at first but becomes slightly elevated later. In complications there is likely to be a left shift in the neutrophils without any change in the lymphocytes.

Psittacosis: In psittacosis there is an absence of leukocytosis at the beginning of the infection. Leukopenia is present in a small number of cases. When the disease is at its height, there is a shift to the right of the neutrophils, accompanied by a distinct fall in lymphocytes. As the patient improves, normal distribution of the blood cells follows. There is a distinct acceleration of sedimentation time at the beginning of infection.

Scarlet fever (scarlatina): The so-called gay blood picture with increase of eosinophils is characteristic of scarlet fever. Most cases show a marked neutrophilic leukocytosis. Values of 20,000/c.mm. in severe cases are often found. In milder cases, the count is around 10,000. The white cells are increased coincident with the exanthematous eruption. With decline in fever the white cells gradually decline. In this disease we find persistent leukocytosis, sometimes for a long time after the decline of the fever. The count is usually back to normal at the time desquamation is finished.

Increase in the eosinophils begins shortly after the onset. In the beginning of the disease, the increase is confined to the neutrophils. The neutrophils show changes in the nuclear structure, with degeneration in the cytoplasm and the formation of vacuoles, and basophilic clumping, the so-called Döhle inclusion bodies. (For further details see remarks on toxic granules, p. 1177.) They are plump and usually heavily stained granules. Monocytes are numerous. There is a relative and absolute increase.

Roth claims that there is a sudden increase of neutrophils in scarlet fever and then a gradual rise to higher levels, with postinfectious lymphocytosis without an increase of monocytes. Plasma cells are sometimes found, according to Ciuca,[640] and the count may be as high as 17%.

The most interesting feature of the scarlatinal blood picture is the eosinophilia. In the first 2 days, the eosinophil count is low, increasing after the second or third day of exanthematous eruption.

According to Türk[641] scarlatina may show as many as 14% eosinophils. Naegeli found as many as 22%. He has one record of 25% in a leukocyte count of 10,000.

The maximum period for eosinophilia usually occurs at the end of the first week. In very severe and in lethal cases, the eosinophilia is absent, according to Ambrus,[642] Georgescu,[643] Markovitch,[644] and Ketschetkoff.[645] Naegeli reports that on the third day of a case of severe septic scarlatina he found only 0.5% eosinophils; on the fourth day the case ended fatally. Heubner and Roth[646] also reported cases of septic scarlatina without eosinophilia.

Basophilic cells also have been found increased in this disease sometimes as high as 4%. A few myelocytes have been found, 3% in a total count of 16,000 having been recorded.

There may be severe secondary anemia in scarlatina. Occasionally nucleated red cells have been observed.

Tuberculosis: Beginning tuberculosis shows an increased total white cell count and a distinct left shift.

The red cells show a slight reduction in total numbers in tuberculosis, except in the terminal stages.

The count of the white blood cells is important in tuberculosis. The discovery by Sabin et al.[647] of the relationship which exists between the monocyte of the circulating blood and the epithelioid cell of the tubercle forms the basis for the increased interest in the cellular mechanism of this disease. Cunningham et al.[648] first showed the importance of the **monocyte-lymphocyte** ratio as obtained from differential counts of the circulating blood. Progressive tuberculous lesions showed a gradually increasing monocyte count and a decreasing lymphocyte count, whereas regressing lesions were accompanied by a reversal of these relationships, i.e., by a lowering of the monocyte-lymphocyte index in the blood (normal is 0.33). Qualitative changes in the monocytes, with the appearance of epithelioid cells in the peripheral blood, are quite as significant as the quantitative changes just mentioned. Medlar[649] has emphasized the importance of the neutrophilic leukocytes in appraising the incipiency and extent of caseation and cavitation in tuberculous foci.

Wiseman[650] re-evaluated certain qualitative changes in lymphocytes with reference to age and maturity which when applied to the study of these cells in tuberculous cases further aids in the analysis and prognosis of the disease. Wiseman found the normal average lymphocyte formula to be 5% young basophilic types, 45% mature moderately basophilic forms, and 50% old, faintly basophilic lymphocytes. In tuberculosis the increase in young immature forms denotes an additional strain placed on the lymphatic tissues. It has been recognized for a long time that resistance to tuberculosis is associated with relative lymphocytosis, but it is essential that the lymphocytes be relatively mature to serve their function. Thus in known tuberculosis patients, if the total lymphocyte count is relatively low, with a degenerative rather than a regenerative shift to the left, the prognosis is grave, even though the monocytic-lymphocytic index may not be markedly increased.

In tuberculosis there is a shift of the neutrophilic leukocytes, with an increase in the stab cells. With increasing severity there is a regenerative blood picture. The commonest type, however, is the degenerative picture. Progressive cases show falling lymphocytosis. Cases doing well show normal lymphocytes or an increase. Eosinophils are usually present, and their presence or absence is of little significance. The same is true of basophils. Allusion has already been made to the ratios between the monocytes and lymphocytes.

Tularemia: The blood picture in tularemia is neutrophilia with a left shift.

Typhoid fever: Typhoid fever is characterized by leukopenia down to 3000-5000/c.mm. and a left shift. Eosinophils disappear at the beginning of typhoid fever. They do not reappear until the third week of the infection.

Certain definite prognostic facts in typhoid fever may be read from the hemogram as follows:

1. A very few eosinophils in the first stage speaks for a mild infection. The reappearance of the eosinophils in the third stage of typhoid fever is a good prognostic factor. A relatively high neutrophil percentage is found in the prodromal stages; as the disease becomes more pronounced, there is neutropenia plus lymphocytosis. Supervening neutrophilia is indicative of a possible complication, especially if there is a regenerative shift. A return of lymphocytes or a regression of the shift in the third stage is a favorable symptom.
2. Unfavorable symptoms are a continued leukopenia and an increasing left shift.

The mechanics of the lowered white count in typhoid fever is undoubtedly a toxic insufficiency of the bone marrow. Studies of the bone marrow in this disease show very few myelocytes and many myeloblasts.

Typhus: In typhus the hemogram is as given in Table 263.

There are many plasma cells. Schilling calls this picture the gay or the colorful blood picture.

Naegeli is inclined to think of slight leukocytosis in typhus. He cites 17 cases by Slatineano with 14,000 leukocytes and 13 cases by Cazeneuve with from 10,000-30,000 leukocytes. Rothacker does not think that the leukocyte count is ever higher than 14,000.

The diagnostic symptoms in the blood picture of this disease are the gay blood picture, with a left regenerative shift. Eosinophils do not disappear quite as rapidly and uniformly as they do in typhoid fever. Cazeneuve places considerable diagnostic importance upon the appearance of myelocytes (2-12%) in typhus.

Most authorities speak of a marked decline in the number of red cells in this disease, accompanied by a hemoglobin drop, sometimes down to 52%. Dychno[651] describes red cell resistance figures up to 0.32-0.36%.

Varicella: There is no change in the hemoglobin and the red cells in this disease. Arneth found during the eruptive period of the disease in a 2 yr. old boy a white cell count of 6000 and with recovery a restitution to normal figures. There is a left shift at the begin-

Table 263

Total white cell count	*Basophils*	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*
8000	0	0	0	5	16	46	27	6

Table 264

Basophils	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*
0	5	1	6.5	8	25	31	23.5

ning, but this rapidly disappears. Nobecourt and Merklen reported 15 patients, ages 1 to 5 yr., without any characteristic changes.

In the suppurative stage there is a characteristic left shift which decreases with the decline of suppuration. So far as the blood platelets are concerned, they decrease with the beginning of fever and increase with the decline of fever.

Variola: The blood picture of variola is characteristic and constitutes a very important link in the diagnostic chain. We find early in the disease a marked regenerative shift (on the fourth day), as shown in Table 264, together with a marked hyperleukocytosis. We are also likely to find many plasma cells, 11% plasma cells having been recorded. According to Schilling there is a marked left shift and an increase in monocytes—not only the normal monocytes are increased, but there also are abnormal large juvenile forms of monocytes. Fischer has described myelocytes and large lymphocytic irritation forms in this disease.

Viral or atypical pneumonia: In contradistinction to bacterial pneumonia, viral pneumonias have leukopenia with a left shift. However, there generally is present moderate lymphocytosis. Owrens et al.[652] showed that in some cases there may occur a very marked absolute and relative lymphocytosis associated with leukocytosis up to 20,000. During this period, such cases may be confused with acute infectious lymphocytosis and with acute infectious mononucleosis. However, the pathologic lymphocytes of acute infectious mononucleosis are either absent or present in only small numbers. The heterophil antibody test is negative.

LEUKOPENIA

Leukopenia is a reduction in the number of leukocytes in the circulating blood below 4500/c.mm. The leukopenic state has been reproduced experimentally and occurs clinically quite frequently. It has been the subject of much investigation.

Lawrence[653] lists 5 possibilities as playing a role in the pathogenesis of leukopenia:

1. Diminished manufacture of white blood cells. This may be the result of (a) simple inhibition, (b) maturation arrest, (c) aplasia of the bone marrow, and (d) infiltration of the bone marrow with foreign cells.
2. Increased utilization of white blood cells. This may occur when large quantities of cells are poured into infected areas, such as in empyema, or when large numbers of cells are lost through normal channels, such as the gastrointestinal tract, the lungs, the spleen, or the liver.
3. Increased rate of destruction in the peripheral blood. This may be due either to abnormal white blood cells or to abnormal substances in the blood channels which cause destruction of the white blood cells, e.g., leukocyte antibodies and substances resulting from sepsis.
4. Redistribution of the white blood cells in the vascular channels, such as occurs in anaphylactic shock following exposure to foreign proteins. Masses of leukocytes are then retained in the lung, liver, and spleen.
5. Hormonal leukopenia. This occurs as a result of hypersplenism, stress, and the administration of ACTH.

Although this summary of the causes of leukopenia is apparently all-inclusive, it is difficult to fortify all these conditions with experimental or clinical facts.

It is thought that viral infections, typhoid fever, malaria, and overwhelming infections can be cited as examples of simple inhibition; nevertheless, there is a possibility that other mechanisms enter into the causation of leukopenia in these conditions. Viral infections, such as influenza, dengue, viral hepatitis, viral pneumonia, acute poliomyelitis, and vaccinia, are almost always accompanied by leukopenia. It is rather interesting that such a large number of viral infections is associated with a low white blood cell count, which is in sharp contrast with the leukocytosis which occurs in most bacterial infections. It might be worthwhile philosophizing that in viral infections leukocytes are not a predominant defense mechanism and are therefore not called upon to handle the infection. Typhoid and malaria (after the attack) are given as examples of simple inhibition, but it is rather interesting that both of these diseases are associated with splenomegaly, a condition which is associated with leukopenic states, perhaps due to some form of hypersplenism.

Maturation arrest of cells of the myelocytic series is most frequently associated with agranulocytosis due to drug intoxication, such as from the sulfonamide drugs. In these instances some hypersensitivity phenomenon has been implicated as the modus operandi of the maturation arrest phenomenon.

Although leukopenia may be seen at times in aplasia of the bone marrow, it is rather surprising how infrequently this phenomenon is associated with rather marked aplasia of the bone marrow in various clinical conditions. Such aplasia may be due to idiopathic states or may result from therapy which destroys the bone marrow. Such therapy includes radiation, both in the form of x-ray and radioactive isotopes, and may be associated as well with severe nutritional deficiencies.

Infiltration of the bone marrow with tumor cells of various kinds includes metastatic carcinoma, multiple myeloma, and other diseases, but a simple explanation is not forthcoming for the leukopenia associated with such states. The relatively simple explanation of overcrowding by tumor cells is hardly acceptable.

When we speak of diminished manufacture of white blood cells within the bone marrow as a cause of leukopenia, we must also take into account the delivery of cells into the peripheral blood. This is a subject about which at the present time we know practically nothing. There are numerous examples of bone marrow overcrowded with white blood cells and with a peripheral leukopenia and vice versa.

Palmer and associates[654] have been able to reproduce in rats a disorder closely resembling the clinical syndrome in man to which the term secondary hypersplenism has been applied. This syndrome, produced by the injection of methyl cellulose in rats, presents a picture characterized by splenomegaly, hyperplasia of the bone marrow, and leukopenia, among other things. In their animals, if splenectomy was performed prior to the injection of the methyl cellulose, no leukopenia occurred; in fact, a splenectomy type of leukocytosis appeared, whereas in animals injected with methyl cellulose and developing the full syndrome, leukopenia was evident. They point out the prophylactic role of the splenectomy in their animals and also state that infiltration of the bone marrow by methyl cellulose-containing macrophages is not the explanation for the pathogenesis of these changes. They particularly point out that pancytopenia failed to develop in splenectomized animals, even though there was an equal degree of infiltration of the bone marrow by the methyl cellulose, and the bone marrows of the animals given the methyl cellulose were characterized by hyperplasia of the blood-forming tissue.

No discussion of the subject of leukopenia could be complete without invoking the present interest in immunologic processes as a cause of anemia, thrombopenia, and leukopenia. Well-documented experimental and clinical studies have been numerous in the literature concerning the role of immunologic abnormalities relating to red blood cells, hemoglobin, and platelets. However, fewer reports are present concerning leukocytes. Kissmeyer-Nielsen[655] carried out transfusion experiments to prove the presence of a leukopenic factor in the blood of patients with leukopenia, and he believed that he could demonstrate such relationship.

In speaking of the leukopenic phases of leukemia, most observers are referring mainly to granulopenia or neutropenia. In acute agranulocytosis unquestionably the disappearance of the granulocytes is due to the failure of the myeloblasts or the myelocytes to mature into adult cells. It has been found that the bone marrow varies according to the stage of the disease, but at times myeloblasts may be the only representatives of the granulocytic series.

Only a few minutes are required to cause the disappearance of granulocytes from the blood after a small dose of aminopyrine in some patients. Vejlens, in Norway, found that certain hydrophil colloids, such as globulin, which produce leukopenia when injected intravenously, do so by causing the white cells to take up a marginal position in the venules of internal organs such as the liver, spleen, and omentum. This is really a type of leukocytic shock because the leukocytes are temporarily out of the circulation. This may be caused by gelatin, globulin, and fibrinogen.

Piney[656] called attention to chronic neutropenia which falls in the diminished manufacture group. Here the only clinical syndrome is apparently causeless fatigue or lassitude, with a granulopenia of less than 200 cells/c.mm. In these cases, treatment, including pentnucleotide, fails to influence this neutropenia although Piney's patient recovered after receiving Hepatex T.

With respect to the fifth possibility, redistribution in the body as a whole, the mechanism that is responsible for the distribution of the white blood cells between the tissues and the vascular channels is unknown. Lawrence gives as the best illustration of this the so-called aleukemic leukemia. In this condition there may be leukopenia, and yet the various tissues in the body may be filled up with white blood cells. That this may occur in other conditions is a distinct possibility.

Many of the facts relating to the cause of leukopenia have yet to be developed.

In summary, leukopenia may be classified (1) according to the location of the defect and (2) according to the main type of cell involved.

Classification

- According to location of defect
  - Diminished formation of leukocytes due to bone marrow defect
    - Radiation effects
    - Effects of drugs and poisons
    - Aplastic anemia
  - Maturation arrest of white blood cells
    - Agranulocytosis
  - Excessive destruction of white blood cells
    - Autoimmune antibodies
- According to main type of cell involved
  - Neutropenia
    - Bacterial and parasitic infections
      - Malaria
      - Typhoid fever
      - Brucellosis
      - Septicemia (overwhelming)
    - Viral infections
      - Measles
      - Hepatitis
      - Poliomyelitis
      - Mumps
    - Blood diseases
      - Aplastic anemia
      - Agranulocytosis
      - Myelofibrosis
      - Pernicious anemia
      - Hypersplenism
    - Toxic agents
      - Radiation
      - Cytotoxic drugs
      - Immune antibodies
    - Hormonal diseases
      - Addison's disease
      - Thyrotoxicosis
      - Acromegaly
  - Eosinopenia
    - Infections before recovery stage
    - Blood diseases
      - Infectious mononucleosis
      - Hyposplenism
      - Aplastic anemia
      - Pernicious anemia
    - Hormonal influences
      - Cushing's syndrome
      - ACTH administration
      - Epinephrine administration
      - Insulin administration
      - Thyroxin administration
      - Stress
  - Lymphopenia
    - Infections
      - Tuberculosis
      - Weil's disease
    - Diseases of lymphatic system and blood
      - Hodgkin's disease
      - Lymphosarcoma
      - Immune antibodies
      - Agranulocytosis
      - Aplastic anemia
      - Bone marrow depression due to radiation effects and effects of drugs and poisons
    - Hormones
      - ACTH
      - Epinephrine
      - Cortisone
      - Insulin

AGRANULOCYTOSIS

This disease has been called by various names. Agranulocytosis was the name given to the disease by Schultz,[657] who first described it. Schilling called the disease malignant neutropenia. It has also been called angina agranulocytica, stomatitis gangrenosa myelophthisica, malignant leukopenia, and sepsis with granulocytopenia.

Definition

Agranulocytosis is a blood disease characterized by the disappearance of neutrophils from the peripheral blood or by a marked diminution. In the pure form of the disease there are no changes in the red blood cells or in the platelets, but there are forms in which the granulocyte-depressing influence also embraces the red cells and the platelets.

Classification

Classification of agranulocytosis is according to whether the disease is congenital or acquired.

- Congenital forms
  - Chronic benign granulocytopenia in childhood
  - Infantile genetic agranulocytosis
  - Neonatal agranulocytosis (transient)
- Acquired forms
  - Toxic-allergic immuno-agranulocytosis
  - Cyclic agranulocytosis
  - Symptomatic agranulocytosis
  - Hypersplenic agranulocytosis
  - Bone marrow depression or obliteration

Neonatal agranulocytosis

Neonatal agranulocytosis is acquired in utero due to transplacental transfer of antileukocytic antibodies. This disease thus forms a bridge between the congenital forms and diseases in the other classification, the acquired forms.

Immunoagranulocytosis (toxic-allergic agranulocytosis)

White blood cells are antigenic. Not only can each type of white blood cell, e.g., neutrophil, lymphocyte, and monocyte, act as an antigen, but also the various parts of each white cell can give rise to antibody formation, e.g., cytoplasm, nucleus, mitochondria, etc.[658, 659] In addition, white blood cells also contain group antigens A and B similar to the erythrocytic antigens. It is interesting to note that experimentally only anticytoplasmic antibodies lead to leukopenia, with the exception perhaps of the so-called L.E. factor. The administration of such an anticytoplasmic antiserum leads to rapid disappearance of the granulocytes in the peripheral blood and to stasis and destruction of the leukocytes mainly in the pulmonary capillaries, but also to some extent in the spleen and liver. The destruction is probably brought about by the same forces that affect sensitized red cells: agglutination, phagocytosis (opsonins?), and lysis (complement utilization?). It has been known for a long time that anaphylactic shock produces leukopenia which is thought to be due to white cell stasis, destruction of leukocytes in the lung capillaries, and the release of heparin, an antileukocytic substance. Experimental allergic reactions (not anaphylactic) fail to produce these events.

Agranulocytosis following administration of certain drugs

One of the most challenging problems in pharmacology is the production of leukopenia following the administration of drugs. The list of drugs capable of producing agranulocytosis in a sensitive patient is almost unending, as can be seen from a quick glance at the list of potentially dangerous drugs published by the American Medical Association.[660]

The condition has been known to follow arsenic and bismuth injections. Madison and Squire[661] reported 13 cases in which they thought this condition was due to the use of benzene chain derivatives. Watkins[662] discussed the role played by barbiturates and aminopyrine in the causation of neutropenic states.

Von Bonsdorff[663] reported 3 cases with agranulocytosis. In 2 cases it was virtually certain that medication with aminopyrine was the cause, and it was probably the cause in the third case. In some persons a few small doses of aminopyrine produce granulocytopenia, whereas in others it appears suddenly some time after large doses of the substance have been administered, without causing disturbance at the time of administration. This shows that therapeutic doses of aminopyrine in sensitive persons may have a detrimental effect on the general condition and on the hematopoietic organs. The action on the granulocytopoietic apparatus is stimulating as well as inhibiting. The total number of granulocytes decreases, but the immature and young forms increase. In some instances leukocytosis may follow. Aminopyrine influences the organs that produce the monocytes and lymphocytes as well as the erythrocytes. The changes disappear spontaneously when medication is discontinued. However, it may be assumed that the continuous use of aminopyrine may eventually cause grave clinical symptoms. Animal experiments proved likewise that the administration of aminopyrine or of related substances may eventually impair the hematopoietic organs and lead to leukocytosis, leukopenia, or anemia.

Kracke[664] pointed out that coal tar products may be responsible for this disease. In one case he claimed that acetophenetidin was probably responsible for this disease. There have been attempts made to differentiate between the etiology of the acute cases and the so-called chronic cases. Roberts and Kracke[665] were impressed by the increasing number of acute fulminant cases and the large number of so-called chronic cases. They claimed that the acute cases represent a very small percentage of the total number of individuals whose blood count is depressed to some degree. Kracke and Parker[666] described the effects of drugs containing the benzene ring on agranulocytosis. They stated that a certain percentage of the cases are due to arsphenamine and a certain percentage to gold salts and perhaps other chemicals, leaving a large group of the idiopathic type with an unknown etiology.

It has been proved that drugs may affect the bone marrow in certain peculiarly sensitive individuals. For instance, purpura following quinine administration has been observed. Benzene administration may be followed by aplastic anemia. Krumbhaar noticed profound leukopenia and marked changes in the bone marrow from mustard gas poisoning.

Pathophysiology of drug-induced agranulocytosis: In a sensitive individual the agranulocytosis-inducing drug acts as a hapten which combines with serum protein to form an antigenically active complex. Antibodies directed against this drug-protein complex are adsorbed onto the granulocytes. When these sensitized cells are exposed to the original drug, they are destroyed in the lung, liver, and spleen. The bone marrow responds to the peripheral cell loss by myeloid hyperplasia and the release of immature granulocytic cells. If the marrow stimulation persists, bone marrow exhaustion may ensue, with resulting complete disappearance of granulocytes. Bone marrow failure can also be explained by assuming that the bone marrow rather than the peripheral cell is the main shock organ, the target for the antibody activity. Although the existence of antileukocytic antibodies has been definitely proved, there is still some difficulty in explaining their specificity (see discussion of autohemolytic anemias).

Agranulocytosis accompanying bacterial and viral infections

The same process of autoantibody production that has been described as being responsible for the production of acquired hemolytic anemias also plays a part in the pathogenesis of agranulocytosis in bacterial and viral infections. The added factor of toxicity must also be taken into consideration. Agranulocytosis may be seen in viral pneumonia, infectious mononucleosis, hepatitis, brucellosis, and measles.

Symptomatic agranulocytosis

Agranulocytosis may be one of the manifestations of lymphomas, leukemias, macroglobulinemia, lupus erythematosus, rheumatoid arthritis, etc. In these diseases the dysproteinemia may be partially responsible for the immune mechanism, as it is in the production of symptomatic hemolytic anemias.

Cyclic agranulocytosis (cyclic neutropenia)

This is a rare disease that occurs in infancy and childhood in which the circulating granulocytes disappear for about 10 days from the peripheral blood at intervals of 3-4 wk. Depletion of marrow granulocytes precedes the peripheral changes.

Symptoms are variable and tend to mimic those seen with agranulocytosis, consisting of stomatitis, acute lymphadenopathy, fever, and fatigue. Sore throats and joint pains may be present. Physical findings are not unusual except for the lymphadenopathy and occasional mild splenomegaly. The white blood cell count varies between 2000 and 4000/c.mm., with neutropenia from 0-16%.

Agranulocytosis due to hypersplenism (splenic neutropenia)

The splenic hyperactivity may be primary or secondary due to splenic enlargement, as seen in cirrhosis of the liver (Banti's disease), portal hypertension, lymphomas, Gaucher's disease, Felty's syndrome, etc. In the etiology a bone marrow-directed hormonal factor may be involved, or hypersequestration of the peripheral cells may take place in the splenic sinusoids.

Chronic agranulocytosis

The name is self-explanatory. The disease may persist for months or years and may or may not be accompanied by clinical manifestations.

Laboratory investigation of agranulocytosis

The disease usually begins with a sore throat of sudden onset. Occasionally, however, there is a history of long-continued sore throat. There are fever and prostration. Examination of the blood will usually suffice to clinch the diagnosis. The hemoglobin and red cell count estimations are normal. Occasionally there may be an unexplained severe anemia, but this is not common. The number of blood platelets may be normal or decreased. The white blood count is the most characteristic feature of the disease.

White blood cell count and differential count

There is a diminution of the total number of white blood cells, which may be down to 1000. We have seen cases with counts as low as 500. The lower the total count, the fewer are the granular elements—so much so that finally no granulocytes are seen at all in the blood spread. The differential count shows complete disappearance of neutrophilic leukocytes with relative lymphocytosis and occasionally monocytosis. In fatal cases the number of white cells remains low and shows a progressive decline. Gradual increase in the percentage of neutrophils and in the number of leukocytes is a favorable symptom. The nuclei of the neutrophilic segmented cells are blurred. The neutrophilic granules appear basophilic and coarse. These are the toxic granules first described by Cesaris-Demel.

Examination of bone marrow

The bone marrow shows variable pictures in different types of agranulocytosis. The picture may be hyperplastic, aplastic, or reticuloendothelial in type. The usual type is aplastic, which has a serious prognosis. A hyperplastic type probably indicates a favorable outcome. In the aplastic type the bone marrow shows many lymphocytes, reticulum cells, and plasma cells. In the hyperplastic type, there are numerous promyelocytes, occasional myeloblasts, and at times metamyelocytes in milder cases. A few of the promyelocytes or myeloblasts are abnormally large and at times binucleated. Regeneration originates in these forms. These abnormal forms point to an abnormality of the cell division rather than to a maturation arrest as the etiologic mechanism of agranulocytosis. The more mature the bone marrow cells are, the better is the prognosis.

Roberts and Kracke[665] saw degenerative myeloblasts in cases of agranulocytosis reported by them. There is a type of reticuloendothelial change in bone marrow in some cases of agranulocytosis which shows marked hyperplasia of the reticuloendothelial system in addition to the presence of lymphocytes and plasma cells in the bone marrow. These reticuloendothelial cells are clasmatocytic in appearance. The erythropoiesis and the thrombocytopoiesis are not involved.

The blood picture that is typical of this disease shows a differential count of, let us say, 90% lymphocytes and 10% monocytes with no granulocytes whatsoever.

Test for leukoagglutinins[667]

Principle: A suspension of leukocytes from normal donors is incubated with the serum to be tested and then examined for agglutination.

Reagents: Inactivate all sera (control serum, group O serum, and unknown serum) at 56° C. for 30 min.

1. Leukocyte suspension.
   - (a) Defibrinate 20 ml. blood from healthy donors (no history of transfusion!) with glass beads in an Erlenmeyer flask.
   - (b) To 4 parts defibrinated blood, add 4% polyvinylpyrrolidone (PVP) solution, mix, and allow to sediment at 37° C. for about 1 hr.
   - (c) Remove the leukocyte-containing supernatant PVP-serum mixture.
   - (d) Count the leukocytes in this mixture and add inactivated group O serum to obtain a concentration of 3000-3500 leukocytes/c.mm. A panel of donor leukocytes should be used, consisting of at least 2 or up to 12 different donor cells.
2. Sedimenting agent.
   - (a) 4% PVP solution in isotonic saline (warm solution to 37° C. to process). Adjust to pH 7.2 with 0.1N NaOH.

Procedure:

1. To 0.1 ml. inactivated unknown serum, add 0.05 ml. leukocyte suspension.
2. Incubate for 1 hr. in a water bath at 37° C.
3. Lyse red cells in the mixture by adding 0.1 ml. 2% acetic acid, mix gently, and allow to stand for 15 min.
4. Examine microscopically (×100) a drop of settled leukocytes from the bottom of the test tube for agglutination.

Treatment of agranulocytosis

Prior to 1942 the chief aim of physicians in treatment of agranulocytosis was to stimulate leukopoiesis by various agents—pentose nucleotide, yellow bone marrow, liver extract, and blood transfusions. These remedies usually proved inadequate, and the mortality rate in agranulocytosis remained high.

In 1942 Dameshek and Wolfson[668] introduced a new policy, i.e., not to worry about the bone marrow but to prevent or control the secondary infection; if infection and toxemia can be controlled, the bone marrow may take care of itself and recover spontaneously. Thus has developed the rationale for the use of sulfonamides and penicillin. Seven cases of agranulocytosis were successfully treated by sulfonamides, but because sulfonamides themselves may induce agranulocytosis, medical authorities turned to penicillin as an equally effective and safe remedy. It was found that penicillin could be used without harm in the presence of granulocytopenia or even of agranulocytosis resulting from severe infections.

In recent years, broad-spectrum antibiotics (tetracycline plus streptomycin, etc.), adrenal corticoids, and ACTH have been added to the physician's armamentarium. If the agranulocytosis is symptomatic in type, the primary disease should also be treated.

INFECTIOUS MONONUCLEOSIS

Historical review

Infectious mononucleosis, which has often been confused with acute lymphocytic leukemia, was first reported in the literature by Filatow[669] in 1885. Later, in 1889, Pfeiffer[670] described it again, calling attention to an infectious process characterized by very definite symptoms to which he gave the name glandular fever. West[671] reported an epidemic of 96 cases limited to children, with no evidence of throat infection. Tidy and Morley[672] reported more cases and described the disease again. They noted the enlargement of the axillary and inguinal glands but not the abdominal signs. Tidy[673] described the epidemic nature of infectious mononucleosis with an incubation period of from 5 to 15 days. Contact infection occurs only in the early stages of the disease, and direct contact or throat droplet infection is probably the mode of transmission. Sprunt and Evans[674] reported cases of this same disease occurring in young adults with infection of the upper respiratory tract with systemic reaction always present, together with the general glandular enlargement with frequent enlargement of the spleen. All cases showed lymphocytosis and moderate increase in the total number of white blood cells, together with a blood picture very much like that found in acute leukemia. There was no hemorrhage or severe anemia in any case reported. Longcope[675] reported cases with acute throat infection but no leukocytosis and very moderate increase in lymphoid cells. Downey and McKinlay[676] reported 9 cases with a study of the hematologic findings. Since 1923 a number of cases have been reported in the literature, together with 2 contributions, one by Bunnell[677] and one by Paul and Bunnell,[678] in which is pointed out a definite laboratory test, known as the **heterophil antibody test,** which will be described later.

Etiology

So far as etiology is concerned, a number of alleged causative organisms have been reported, notably *Listeria monocytogenes,* isolated from rabbits by Murray,[679] and from an apparent case in man by Nyfeld.[680] Other organisms have been described but have not been definitely established. Many believe this to be a viral disease, but all attempts to cultivate a virus have failed so far. It has been suggested that the virus, similar to the cold virus, must require very special growth conditions in vitro.[681]

Laboratory diagnosis

Lymph node biopsy

Examination of the biopsy material from lymph nodes of patients with infectious mononucleosis shows that the hyperplasia of lymphocytes is not as extensive as in cases of lymphadenosis. The atypical structure of the lymphomata and the increase in their number indicate that the disease is due to infection with some organism or virus which has a very specific stimulating effect on the lymphocytes and reticulum and a depressing effect on the granulocytic system.

Blood picture

There is no particular change in the number of red cells, the amount of hemoglobin, or the number of blood platelets in infectious mononucleosis. Leukopenia is striking in the beginning. There is usually leukocytosis coincident with enlargement of the lymphatic glands. The differential count shows a high lymphocyte count, often reaching a very high level, with the appearance of immature forms (Fig. 446). Downey and McKinlay[676] made an exhaustive hematologic study. They divided their patients into 3 groups, entirely dependent upon the type of atypical lymphocytes present in the blood picture.

Type I: The nucleus is oval, slightly lobulated, and contains a coarse mature chromatin. The cytoplasm is pale to dark blue, vacuolated (toxic), and abundant. Azurophilic granulation is often absent, but it may be prominent.

Type II: The cells are larger; the nucleus is round or oval and shows a mature chromatin pattern. The cytoplasm is large and pale and does not appear to be spongy. Other cells in this category have a somewhat plasma cell-like appearance due to a somewhat hyperchromatic eccentrically situated nucleus.

Type III: In this group are cells which are more immature, with deeply basophilic cytoplasm. Azurophilic cytoplasmic granules have been described in some of these cells. The nucleus is adult in type and round or oval.

These atypical cells appear in the peripheral blood from the fourth to fourteenth day, but their division into types serves no clinical purpose. Similar cells, virocytes, can be found in many viral diseases, such as hepatitis, upper respiratory viral infections, measles, viral pneumonia, herpes zoster, and herpes simplex. There is also some evidence that these cells do not represent a specific response to viral stimuli since they can also be seen whenever allergic and hormonal stimuli produce lymphocytic changes.[682] Recent electron microscopic studies of the leukocytes in infectious mononucleosis have failed to shed light on the nature of these cells.

The peroxidase test on these cells is negative, which would incline one to believe in their lymphocytic origin. Osgood[683] directed particular attention to the nuclear fenestrations in the mature small lymyphocytes of this disease. These fenestrations are multiple holes and are considered by Osgood of definite diagnostic importance. The hemograms shown

Table 265

Date	*Basophils*	*Eosinophils*	*Myelocytes*	*Juvenile cells*	*Stab cells*	*Segmented cells*	*Lymphocytes*	*Monocytes*	*Plasma cells*
6/19/35	0	1	0	½	2½	25	62½	8½	0
6/21/35	1	5½	0	½	6	28	73	2½	½
6/22/35	0	1½	0	0	1½	18	76	2½	½
6/24/35	1½	1½	0	0	3	27	64	3	0
6/25/35	1	½	0	0	3½	28	62½	3½	1
6/26/35	0	1½	0	0	4½	23½	65	5½	0
7/ 3/35	1	4	0	0	4	35	53	3	0

in Table 265 from a case of infectious mononucleosis illustrate the hematology of the disease.

Serology

Heterophil antibody test

Because of the difficulty in making an absolute diagnosis on clinical and cytologic grounds, the suggestion of a serologic diagnostic test, proposed by Paul and Bunnell[678] in 1932, was hailed with a great deal of satisfaction. This test is based on the presence of sheep heterophil antibodies in the blood of the patient. The heterophil antibody concerned may be defined as an antibody reacting with an agent other than and phylogenetically unrelated to the specific antigen responsible for the production of the antibody in question. The presence of increased sheep heterophil antibodies in the blood in patients with infectious mononucleosis was rapidly confirmed by a number of independent investigators.

Presumptive test

The following technic for the heterophil antibody test is adapted by Kracke[684] from Davidsohn, Bernstein, Paul, and Bunnell.

Procedure: Eleven tubes are required for the test —10 for the serial dilutions of the patient's serum and the eleventh for a control over saline and sheep cells used.

1. To tubes 2, 3, 4, 5, 6, 7, 8, 9, 10, and 11 add 0.5 ml. physiologic saline. Tube 1 receives no saline.
2. Pipet 0.5 ml. patient's inactivated serum (heated to 55°-56° C. for 15 min.) into tube 1. The serum is heated to inactivate complement to prevent the action of possible anti-sheep cell hemolysins.
3. Place 0.5 ml. of patient's inactivated serum in tube 2.
4. Mix the contents of tube 2 carefully by drawing in and out of the pipet, and transfer 0.5 ml. to tube 3.
5. Mix the contents of tube 3 in the same manner and pipet 0.5 ml. into tube 4; then continue in this manner through tube 10.
6. Mix the contents of tube 10 and discard 0.5 ml. Tube 11 receives no serum.
7. Dilutions of serum in the 10 tubes are now as follows: (1) undiluted, (2) 1:2, (3) 1:4, (4) 1:8, (5) 1:16, (6) 1:32, (7) 1:64, (8) 1:128, (9) 1:256, and (10) 1:512.
8. To each of the 11 tubes add 0.5 ml. 2% suspension of sheep cells. The sheep cells are prepared in physiologic saline. Wash 3 times immediately before use by adding 3 times their vol. physiologic saline and centrifuging until firmly packed, removing the supernatant fluid each time before adding fresh saline. After the last centrifuging remove the saline and make a 2% suspension by adding 49 vol. saline to the packed sheep cells.
9. After the addition of the sheep cell suspension, add 1 ml. saline to each of the 11 tubes. The total quantity in each tube is now 2 ml.
10. Shake the tubes thoroughly and let stand at room temperature for 2 hr. Initial reading may be made after 15 min.

Report the highest dilution which shows macroscopic agglutination after gently shaking the tubes to resuspend sediment. The titer is the reciprocal value of the highest serum dilution showing agglutination.

There are many factors that limit the usefulness of this test. Forssman in 1911 described a nonspecific antigen which is present in the tissues of various animals, such as the guinea pig, horse, rabbit, and pig, but is absent in man, sheep, etc. If horse serum, for instance, is injected into patients, antibodies acting on sheep erythrocytes will appear. Such so-called heterophil antibodies are present in normal persons and may reach a titer of 1:64. This titer may rise in viral infections, allergies, and certain blood diseases such as polycythemia, acquired hemolytic anemia, myelogenous leukemia, etc. The heterophil antibody test is therefore nonspecific and is usually referred to as a **presumptive test.** It is considered to be positive if agglutination occurs in a dilution of 1:64.

Sheep heterophil antibodies similar in character to those found in infectious mononucleosis had been previously demonstrated by Davidsohn[685] and many others in the blood of individuals after injections of native or therapeutic horse serum, particularly when serum sickness developed. Davidsohn[686] has also shown that such antibodies might persist in the blood of a patient for a year or more after treatment with horse serum.

Differential absorption test

Stuart et al.[687] found that the sheep antibodies in serum sickness were completely adsorbed by emulsions of guinea pig kidney, whereas those of infectious mononucleosis were not, proving that the heterophil antibody in infectious mononucleosis is not a pure anti-Forssman-antigen-antibody, as the latter is completely adsorbed by any Forssman antigen such as guinea pig kidney. On the other hand, the heterophil antibody in infectious mononucleosis is completely adsorbed by beef erythrocytes.

The patient's serum should be titrated for antisheep agglutinin in 3 ways: (1) unadsorbed, (2) after adsorption with guinea pig tissue, and (3) after adsorption with beef tissue.

Equipment:

1. 24 test tubes, 75 × 12 mm.
2. 1 test tube rack.
3. 4 pipets, 0.25 ml.
4. 2 pipets, 0.50 ml.
5. 2 pipets, 1 ml., graduated in 0.1 ml.
6. 2 sterile syringes, 1 and 2 ml.
7. 2 sterile needles, 20 gauge.
8. 2 dropper pipets.
9. 2 centrifuge tubes, 15 ml., graduated.
10. 1 water bath regulated at 56° C.
11. 1 centrifuge.
12. 1 clock or watch.
13. 1 viewing box such as is used for blood grouping tests.

Reagents:

1. Sodium chloride solution, 0.85%.
2. Patient's serum.
   (a) Obtain in the usual manner and inactivate in a water bath at 56° C. for 30 min.
3. Sheep red blood cells, 2%.
   (a) Make either from freshly secured blood or prepare from preserved sheep cells.
   (b) Place 1.5 ml. well-mixed suspension of sheep cells into a 15 ml. centrifuge tube.
   (c) Wash 3 times in saline or until the supernatant saline is clear and colorless. This should leave about 0.3 ml. packed sheep cells. Dilute 0.3 ml. sheep cells with saline to 15 ml. to make a 2% suspension.
3. Guinea pig kidney antigen. This can be obtained ready for use.*
4. Beef cell antigen. This can be obtained ready for use.*

Procedure:

Titration of unadsorbed serum:

1. Place 6 test tubes in a test tube rack.
2. Place 0.4 ml. saline in the first tube.
3. Place 0.25 ml. saline in each of the other tubes.
4. Add 0.1 ml. patient's inactivated serum to the first tube.
5. Mix by drawing into and out of a pipet several times.
6. Transfer 0.25 ml. from the first to the second tube, and mix. Transfer 0.25 ml. to the next tube and mix.
7. Continue transferring and mixing through tube 6. This leaves 0.5 ml. in tube 6. Remove and discard 0.25 ml. from tube 6.
8. Add 0.1 ml. of washed sheep cell suspension to each tube.
9. Shake well and let stand at room temperature for 2 hr.
10. Examine for clumping of the red blood cells after gently shaking the tubes.

According to Davidsohn (adapted from Stuart and co-workers), the final dilutions are 1:7, 1:14, 1:28, 1:56, 1:112, etc. (This apparently includes the quantity of sheep cells used: first tube contains 0.05 ml. serum in a total of 0.35 ml.)

The titer is the weakest dilution of the serum which gives clumping that is visible to the naked eye.

Titration of serum adsorbed by guinea pig antigen:

1. Shake the suspension of guinea pig antigen thoroughly and place 1 ml. in a test tube.
2. Add 0.2 ml. inactivated serum.
3. Shake the tube well and centrifuge at high speed for 5 min.
4. Transfer the supernatant liquid with a capillary pipet to a clean test tube. This is the adsorbed serum.
5. Place 0.25 ml. saline in each of 6 tubes.
6. Add 0.25 ml. adsorbed serum to tube 1.
7. Mix and transfer serially 0.25 ml. to each tube; discard the last 0.25 ml. from tube 6.
8. Add 0.1 ml. washed sheep cells to each tube.
9. Shake well and let stand at room temperature for 2 hr.
10. Examine for clumping of the red cells after gently agitating the tubes.

The final dilutions are 1:14, 1:28, 1:56, etc. (includes the added sheep cells).

The titer is the weakest dilution of serum which gives clumping visible to the naked eye.

Titration of serum adsorbed by beef cell antigen: Proceed exactly as for guinea pig antigen, using thoroughly shaken beef blood cell antigen in place of the guinea pig antigen.

Interpretation of results

Beef cell antigen adsorbs the infectious mononucleosis (I.M.) antibody completely, whereas guinea pig antigen does not. For example, if the titer with unadsorbed serum is 1:56, and the patient has infectious mononucleosis, the titer after beef cell adsorption will be 0 or nearly 0, whereas after guinea pig adsorption it will be 1:56, 1:28, or even 1:14, but no lower.

The titer of the presumptive test is less important than the adsorption results.

It must be remembered that the specific infectious mononucleosis antibodies are not pres-

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*An excellent preparation of guinea pig kidney antigen and beef red cell antigen is available from Certified Blood Donor Service, Jamaica, L. I., N. Y.

Table 266. *Differential adsorption of heterophil antibodies*

	Adsorption by guinea pig kidney	*Adsorption by beef erythrocytes*	*Adsorption by sheep erythrocytes*
Normal serum	Yes	No or partial	Yes
Hepatitis	Yes	Yes	Yes
Serum sickness or viral diseases	Yes	Yes	Yes
Infectious mononucleosis	No or slight	Yes	Yes

ent throughout the disease, and they should be sought on more than one occasion if the clinical symptoms and the blood findings are suggestive of infectious mononucleosis.

Very recently (1961) Tomcsik[688] described a new serologic test for the diagnosis of infectious mononucleosis using trypsinized beef erythrocytes and only 5 test tubes. This test appears to be as specific as the Davidsohn test but is much shorter and less complicated.

ACUTE INFECTIOUS LYMPHOCYTOSIS

Carl H. Smith reported[689] an account of a new disease entity, acute infectious lymphocytosis. He reported 2 cases in 1941 and saw 4 cases during March, 1943. The fact that 3 of the patients were members of one family and 1 a hospital contact impressed him with the possible communicability or infectiousness of the disease. Other cases have been reported at various hospitals in New York City where these first cases were noted.

This is a disease with an elevated leukocyte count persisting for approximately 3-5 wk. The clinical course varies in intensity. The disease has a possible incubation period of 12-21 days and may be marked by varying degrees of constitutional reaction at the onset, such as vomiting, irritability, and fever, together with acute abdominal signs and symptoms and occasionally signs of involvement of the nervous system. There is no lymphadenopathy nor is there a palpable spleen. The heterophil agglutination reaction is uniformly negative. In 2 of the cases the biopsy of a lymph node showed strikingly similar microscopic appearance. An oustanding feature in these biopsies was a striking proliferation of the reticuloendothelium of the sinuses, which were blocked by masses of cells. In addition, the lymph follicles showed a number of changes. Some of the germinal centers showed a hyaline degeneration of varying degree. Many of the follicles were notably inconspicuous, and in some sections they were practically obliterated. A feature common to most of the cases was infection of the upper respiratory tract.

The striking feature of these cases was the hyperleukocytosis with relative and absolute lymphocytosis persisting for a prolonged period. Normal small lymphocytes accounted for this increase and presented the most important diagnostic element of the blood picture.

The differential diagnosis from leukemia was based largely on the absence of lymphoblasts. Lymphoblasts are usually round, large, and uniform in size and show a distinct nuclear pattern. Besides, in acute lymphocytic leukemia with a hyperleukocytosis of the magnitude found in infectious lymphocytosis the blood would show a severe anemia and a decrease in platelets, and enlargement of the spleen and lymph nodes would be noticed.

Examination of the bone marrow also serves to differentiate between leukemia and this disease. In acute lymphocytic leukemia in children, marrow aspiration shows complete replacement of the bone marrow by lymphoblasts. In acute infectious lymphocytosis, on the other hand, the myeloid and erythroblastic elements are present in normal proportions and the only abnormality is the presence of normal lymphocytes in increased percentage.

In the first case the first blood count showed 45,000 white cells/c.mm., 45% neutrophils, and 48% lymphocytes. In a few hours, the white cell count rose to 55,400 with 72% lymphocytes. The total count in some cases ran as high as 68,300, and the lymphocytosis amounted to as much as 90% in one case. The elevated blood levels persist from 3-5 wk.

The normal lymphocytes in this disease are in sharp contrast to the atypical and abnormal lymphocytic elements in infectious mononucleosis and to the lymphoblasts in leukemia.

The heterophil agglutination reaction was uniformly negative in these cases, and the serologic reactions were negative for lymphocytic choriomeningitis and for influenza A and B virus.

In all cases the disease was uncomplicated and had a uniformly favorable outcome.

LUPUS ERYTHEMATOSUS (L.E.) CELL PHENOMENON[690]

The discussion of the lupus erythematosus factor (L.E.) follows the discussion of agranulocytosis because during the last decade the causative factor has been identified as an autoantibody.

The often bizarre clinical patterns observed in disseminated lupus erythematosus have made the diagnosis of this disease difficult, especially when the typical skin manifestations are absent. A diagnostic aid was added with the finding of the L.E. cell in bone marrow by Hargraves et al.[690] These bone marrow findings were soon verified,[691, 692] and others[693, 694] were able to find these cells in the peripheral blood of patients with lupus erythematosus. Since the original description of this unusual cell, it has been shown that the serum (or plasma) of patients with lupus erythematosus will reproduce the phenomenon when added to the bone marrow[693, 695] or the blood[696, 697] of normal persons or of patients suffering from diseases other than lupus.

These observations led to the conclusion that the abnormal factor was present in plasma, and Haserick[698] identified its association with the gamma globulin fraction. Kurnick et al.[699-701] have reported that in addition to the specific factor present in fraction II of L.E. sera, other elements participate in the phenomenon, namely (1) deoxyribonuclease, (2) an inhibitor of deoxyribonuclease, and (3) a factor or factors in fraction III of normal and L.E. sera. Kurnick et al.[702] have followed these ideas with determination of the susceptibility of leukocytes from different animal species to alteration by lupus erythematosus serum. These observers found that the leukocytes of the chicken and horse were the most susceptible and that those of the guinea pig and dog were highly susceptible. In all these species, the demonstration of L.E. cell formation was much more striking than when using human leukocytes, and they recommend an animal substrate of leukocytes for performing the L.E. cell test.

Very early in the observations on the L.E. phenomenon it was realized that the abnormal cells were not dependent on anticoagulant, and it was possible to demonstrate L.E. cells in sections.[703, 704]

Table 267. *Means of differentiating some conditions characterized by lymphocytosis*

	Pertussis	*Aleukemic leukemia*	*Chronic lymphocytic leukemia*	*Acute lymphocytic leukemia*	*Acute infectious lymphocytosis*
White cell count	May be 40,000 or higher	Normal or subnormal	Very high; 50,000 and up	Extremely high; up to 1,000,000	Very high; 45,000-70,000
Red cell count	No significant change	Low	Low	Very low	Usually normal
Hemoglobin	No significant change	Low	Low	Very low	No significant change
Number lymphocytes characteristically present	60-80%	About 60%	As many as 90-100%	Up to 100%	45-90%
Size of lymphocytes	Small	Large	Small	Large	Mostly small
Pathologic or special forms of lymphocytes	—	Lymphoblasts; Rieder forms; atypical lymphocytes; plasma cells	Lymphoblasts sometimes found; plasma cells	Lymphoblasts: many atypical; plasma cells	None
Pathologic red cells	—	Normoblasts; polychromasia; other nucleated reds may be seen	Normoblasts; polychromasia	Normoblasts and other nucleated reds; pathologic forms; polychromasia, etc.	None
Blood platelets	No significant change	Low	Low	Low or absent	Normal
Bone marrow	—	Lymphatic	Lymphatic	Lymphatic	Normal except for lymphocytosis

The finding of L.E. cells in the peripheral blood or bone marrow of any patient imposes a diagnosis of lupus erythematosus although a few exceptions to this statement are to be found: individual cases of dermatitis herpetiformis,[705] pernicious anemia,[705] leukemia,[706] multiple myeloma,[707] and primary amyloidosis.[704] Haserick[708] contaminated plasma samples with fungi and simulated the L.E. cell phenomenon. Walsh and Zimmerman[709] have reported 3 cases of severe penicillin reaction in which L.E. cells were found in the bone marrow and the plasma factors in the plasma. A lupuslike syndrome has been described in patients treated with hydralazine (Apresoline) for hypertension,[710] in which L.E. cells can be demonstrated. The phenomenon disappears after withdrawal of the drug. Despite these few exceptions, the finding of L.E. cells should be strong evidence of the diagnosis of lupus erythematosus unless proved otherwise. On the contrary, failure to demonstrate such cells does not militate against such a diagnosis.

L.E. cells are most abundantly present in the acute cases of lupus erythematosus and are particularly easy to demonstrate in the terminal phases of the disease. The finding of L.E. cells in patients in remission is variable. At times there are inexplicable variations in numbers of L.E. cells. Occasional patients are seen whose preparations contain numerous L.E. cells on one day and only rare forms the following day.

As with many other laboratory searches, the incidence of positive L.E. preparations is proportional to the vigor with which the pursuit is made, and often many preparations must be made on a given patient before these cells are found.

The positive L.E. test exhibits 3 phenomena: (1) the L.E. cell, (2) rosettes, and (3) the so-called free Klemperer (hematoxylin) or nuclear bodies. The last 2 phenomena are helpful in the diagnosis of L.E., but only the first is diagnostic. L.E. cells are usually neutrophils which contain within their cytoplasm

Infectious mononucleosis	*Agranulocytosis*	*Idiopathic aplastic anemia*	*Pernicious anemia*	*Endocrine disturbance*	*Chronic tuberculosis*
Normal or slight leukocytosis	Very low, below 2500; may be below 1000	Progressively lowered; may be below 500	Low; average around 3000; may be lower	High normal	High normal
No significant change	Normal or slightly lowered	Progressively lowered; may be below 1,000,000	Low, around 1,500,000-3,000,000	No significant change; usually lowered	No significant change
No significant change	Lowered	Progressively lowered	Low	Often lowered	No significant change
Around 60-70%	May be as high as 90-100%	May be normal or up to 100%	Around 40-50% or higher	About 40-45%	40-50%
Large	Small	Very small	Large	Large	Large; size depends upon amount healing
Types I, II, and III special forms seen in this disease; plasma cells	—	—	—	—	—
None	None	None; as a rule not even polychromasia	Megaloblasts and megalocytes; macrocytic hyperchromic erythrocytes; polychromasia, etc.	Polychromasia; sometimes basophilic punctation; occasional normoblasts	No significant change
No significant change	Normal	Low relative and absolute counts; may completely disappear	Low absolute counts	No significant change	No significant change
	Aplastic type or packed bone marrow	Fatty degeneration or infiltration with small lymphocytes	Megaloblastic	—	Left shift

large homogeneous, amorphous, violaceous inclusions. If the inclusions are atypical and more or less retain the nuclear chromatin pattern, we call these cells **tart cells** after a patient named Tart in whose blood Hargraves found these cells. They are pseudo-L.E. cells, are not diagnostic of L.E., and represent phagocytosis of hypersensitivity. Rosettes are clusters of neutrophils surrounding amorphous nuclear material. The free Klemperer bodies are free amorphous nuclear material.

The formation of the L.E. cell occurs in 2 phases. The first step is the action of a plasma factor (L.E. factor) on white cell nuclei which lose their chromatin pattern and are released from the disintegrating cell. In the second step, these freed and altered nuclei are phagocytosed by uninjured white cells. The L.E. factor is able to act on the nuclei of any of the white cells in the peripheral blood or bone marrow. The phagocytosing cells comprise mainly polymorphonuclear neutrophilic cells but occasionally also eosinophils, basophils, and monocytes.

The L.E. factor is a gamma globulin. Its activity can be neutralized by antihuman gamma globulin serum. It is present not only in the plasma of L.E. patients but also in other protein-containing fluids, such as exudates, transudates, and protein-containing urine. The L.E. factor crosses the placenta and produces a transitory L.E. cell phenomenon in newborn babies of affected mothers. Goodman[711] showed that under the influence of the L.E. factor the nucleus imbibes water and protein so that it swells. The imbibed protein replaces histon in the nuclear deoxyribonucleic acid, producing the characteristic homogeneous amorphous staining quality of the affected nucleus.

What is the L.E. factor? Based on the action of antileukocytic antibodies (leukoopsonins) and their production of nucleophagocytosis, it has been suggested that an immune serum is responsible for the L.E. phenomenon.

With the help of immune electrophoretic technics and the single and double diffusion technics of Oudin and Ochterlony, it has been established that the L.E. factor is an antinuclear antibody produced in response to nucleoprotein.[712, 713, 715] This may not be the entire story. Bensze has repeatedly shown evidence of the existence of transferable and nontransferable L.E. plasma factors. The antinuclear L.E. factor fulfills most of the criteria of antibody except that of specificity since it attacks cells of widely separated species. It also overlaps with antibodies found in other diseases, such as rheumatoid arthritis, Hashimoto's disease, and hepatitis.

Laboratory diagnosis of lupus erythematosus

Hematologic findings

Red blood cells: There is usually a normocytic normochromic anemia which may be due to hemolysis or to bone marrow depression. If the anemia is hemolytic, the Coombs test is frequently positive.

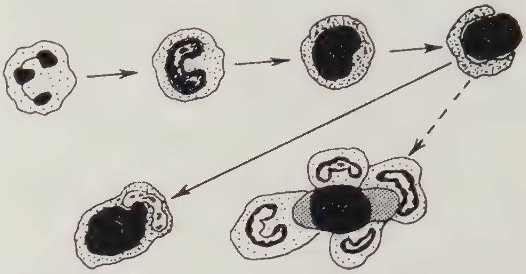

Fig. 437. Outline of nuclear lysis and formation of L.E. cell and rosette. (Modified from Robineaux, R.: Étude microcinématographique en contraste de phase du mécanisme du phénomène LE. In Grabar and Miescher, editors: Immunopathologie, Symposium, Basel, 1958, Benno Schwabe & Co.)

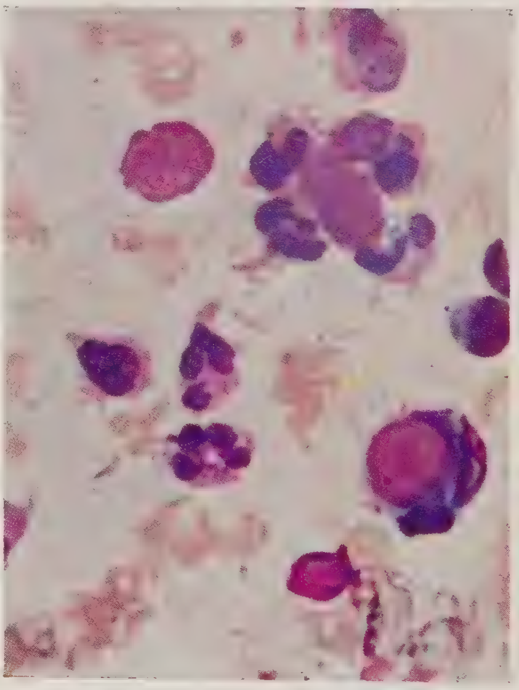

Fig. 438. Lupus erythematosus (L.E.) cells. Cluster in upper right-hand corner is a typical rosette with an L.E. body in the center. The cell containing a body in lower right-hand corner is a tart cell, which is not pathognomonic of lupus erythematosus. Source of material, bone marrow. (×800.)

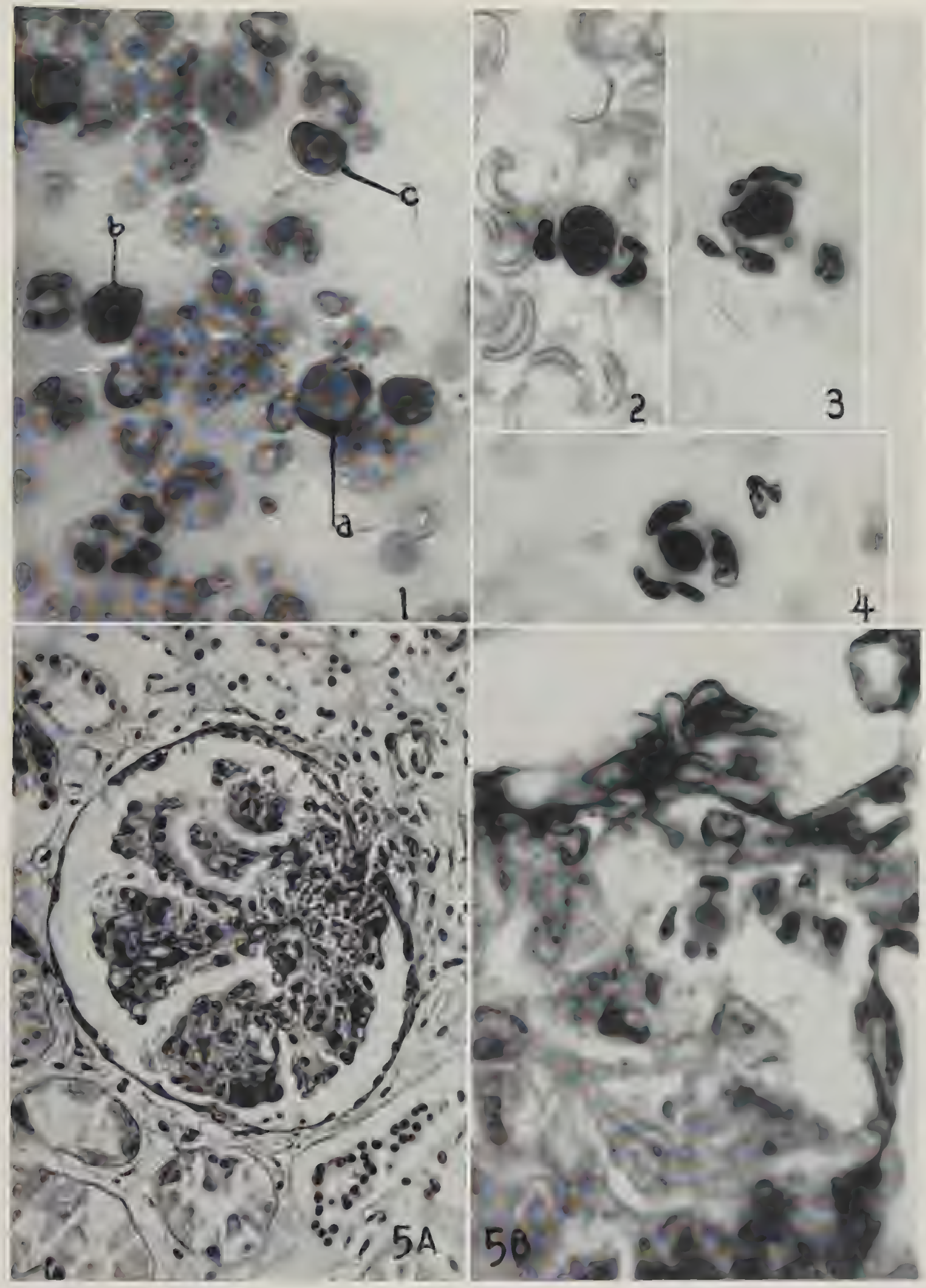

Fig. 439. Lupus erythematosus (L.E.) cell phenomenon. Bone marrow spreads and tissue sections. **1,** Spread of sternal marrow aspirate. An intracellular, **a,** and an extracellular, **b,** L.E. body are seen in addition to a degenerated nucleus, **c.** (Wright-Giemsa stain; ×940.) **2,** Paraffin section of the sternal marrow aspirate with an L.E. cell. (H & E stain; ×1530.) **3,** Paraffin section of the sternal marrow aspirate with an L.E. cell. Tissue has been treated with ribonuclease and stained with toluidine blue. L.E. body stains similarly to the nuclear material. (×1530.) **4,** Paraffin section as in **3.** Tissue has been stained by the Fuelgen method; L.E. body stains similarly to the nuclear material. (×1530.) **5A,** Paraffin section of kidney of a patient with clinical and autopsy diagnoses of disseminated lupus erythematosus. Sections have been treated with hyaluronidase and stained by the Hotchkiss method. Intracellular and extracellular L.E. bodies were seen. (×280.) **5B,** Higher power of **5A,** showing a typical L.E. cell. (×1530.) (From the collection of H. Agress.)

White blood cells: There is usually leukopenia unless infection is responsible for the leukocytosis. Eosinophilia is rarely seen.

Platelets: Thrombocytopenia is frequent and is responsible for hemorrhagic manifestations such as purpura. In any patient who appears to have idiopathic thrombocytopenic purpura, lupus erythematosus should be excluded.

Methods for demonstrating L.E. cell

Clot method

1. Withdraw 7 ml. venous blood, allow to clot and discard serum.
2. With a pestle grind the clot through a fine wire mesh screen.
3. Discard the fibrin left on the screen and collect the sieved portion.
4. Distribute the latter in Wintrobe sedimentation tubes and centrifuge at 1000 r.p.m. for 10 min.
5. Make smears of the buffy coat only and stain with Wright stain.
6. Examine for L.E. cells with high dry magnification.

Rapid slide screening test

This test employs a reagent prepared from polystyrene latex and deoxyribonucleic protein.* The test is new (1961). It is hoped that it will solve the frequent difficulty of distinguishing rheumatoid arthritis from L.E. Rothfield et al.[716] in a recent series consider this test superior to the L.E. test.

Our own experience and that of Dubois[717] differ from Rothfield's inasmuch as, although we admit that the test is specific, we do not consider it sensitive enough to be used as a screening procedure.

Other tests for L.E. cells

Mudrick and others[718] described a capillary L.E. cell test which does not require special equipment but can be done in microhematocrit tubes and has the advantage of incubation of the plasma L.E. factor with mainly leukocytes.

Rebuck et al.[719, 720] showed that a patient who has L.E. may form L.E. cells in his own skin window either spontaneously or after addition of his (patient's) own plasma to the skin window.

Nonhematologic findings

Plasma proteins: There is an increase in the globulin fraction (hypergammaglobulinemia) with or without depression of albumin, depending somewhat on the state of the kidneys. The increase in fibrinogen and in gamma globulin is responsible for the increased sedimentation rate in L.E. These protein changes may also be responsible for positive flocculation tests even without liver disease, for the high heterophil antibody titer, and for the falsely positive serologic tests for syphilis.

Urine: Proteinuria is found if the kidneys are involved.

Synovial and pleural fluids: These fluids may contain L.E. cells.

L.E. is a protean disease which may affect almost any body system, but the present discussion is confined to those manifestations which can be detected in the laboratory.

SUMMARY OF LEUKOCYTIC CHANGES IN VARIOUS PATHOLOGIC CONDITIONS

The following summary offers a recapitulation of the changes in the Schilling differential count seen in various pathologic conditions.

Leukocytosis with increase in basophils:

1. Myelocytic leukemia.
2. Polycythemia vera.
3. Serum injection.
4. Hemophilia.
5. Convalescence from beriberi.
6. After multiple blood transfusions.
7. In many conditions customarily accompanied by eosinophilia.

Eosinophilia with increased white cell count:

Note: It is a biologic rule that severe irritations do not cause weaker or paradoxical reactions; i.e., the same processes which in moderate degrees may produce eosinophilia in greater degrees cause either hypereosinophilia or aneosinophilia.

1. Exudative processes: bronchial asthma, exudative diathesis and exudative intestinal catarrh of children, colitis membranacea, Quincke's edema, etc.
2. Anaphylaxis and anaphylactoid conditions: serum disease, hay fever, urticaria, migraine, idiosyncrasies, etc., and irritations of the vagus nerve.
3. All diseases due to worms wherever located (eosinophilia may dwindle to aneosinophilia [insufficiency] because of overinfestation): trichinosis, schistosomiasis hepatica, ancylostomiasis, bilharziasis, filariasis, *Diphyllobothrium latum* infestation, echinococcosis, and intermittently with all intestinal parasites.
4. Skin diseases: psoriasis, pemphigus, urticaria, eczema, herpes zoster, impetigo contagiosa, some dermatitides, prurigo hebrae, premykotic exanthema, mycosis fungoides, purpura.
5. Scarlet fever beginning on the third day of the eruption.
6. Gonorrhea, especially in females.
7. Tumors occasionally, especially those of the lungs and peritoneum; rectal carcinoma, especially in serous metastases.
8. Nephrosis or nephritis occasionally (slight affections).

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*Hyland Laboratories, Los Angeles, Calif.

9. Muscular rheumatism (Bittorf).
10. Leukemia. This serves to differentiate leukemia from leukemoid pictures.
11. Pernicious anemia in the beginning, during remissions, and under liver therapy.
12. Convalescence from infections (good sign).

Decrease or absence of eosinophils (considered an unfavorable symptom):

1. Overirritation of eosinophilic processes, particularly in severe affections.
   (a) Due to worms.
   (b) Height of serum disease.
   (c) Pernicious anemia in severe stages.

Normal total neutrophils and low white blood cell count:

1. Severe pernicious anemia (this usually shows neutropenia).
2. Severe aplastic anemia (this usually shows neutropenia).
3. Osteoscleroses and diseases with reduction of bone marrow.

Neutrophilia without shift but with increased white blood cell count:

1. During digestion.
2. Muscular activity.
3. Spasmodic conditions.
4. After hemorrhages.
5. Polycythemia (at times a slight shift).
6. Very superficial or benign infections.
7. Simple intestinal catarrh.
8. Nephritides.
9. Abortive cases of infectious disease.
10. Polyarthritis acuta (often shows a right shift, an acute infection without a left shift).
11. Tetanus.
12. Struma.
13. Uncomplicated malignant tumors.
14. After sodium chloride infusions temporarily.
15. After certain medicaments.
16. During violent internal or external hemorrhages.

Neutrophilia with slight stab shift with high normal or increased white blood cell count:

1. Mild cases of acute infections and protozoal diseases: anginas, catarrhs, and malaria during attacks.
2. Severe diseases of the digestive system: intestinal catarrhs and liver diseases including jaundice in which the epithelia are alone involved.
3. Superficial, benign, or encapsulated infections.
   (a) After operations.
   (b) After incision of abscesses.
   (c) Suppurative wounds without general involvement.
   (d) Slight catarrhal appendicitis.
   (e) Open suppurations of eyes, ears, and pharynx.
   (f) Appendicitis, gynecologic, and liver pus collections, entirely encapsulated.
   (g) Chronic diseases with neutrophilic irritation (septicemias and endocarditis).
   (h) Broken-down ulcerative tumors.
   (i) Mild active tuberculosis.
   (j) Tuberculous abscess formation.

These slight shifts are important for the following reasons:

1. To determine infectious processes.
2. To estimate the latent continuance of apparently healed infections or the danger of relapse.
3. To outline as relatively harmless acute illnesses or exacerbations of severe conditions which have been reactivated.

Neutrophilia with regenerative shift and increased white blood cell count:

1. Very violent exercise (marathon races).
2. Diabetic coma.
3. Pregnancy.
4. Severe cases of many acute infectious diseases: pneumonia, senile pneumonia, diphtheria, scarlet fever, cholera, bacterial dysentery, erysipelas, meningitis epidemica, typhus, and variola.
5. Acute protozoal disease during attacks.
6. All acute and progressive internal infections: beginning empyema, newly formed abscess, appendicitis destructiva et perforata, peritonitis, purulent inflammation of bile ducts and urinary passages, pus infection of bone marrow, encapsulated malignant pustular affections of ears, eyes, and pharyngeal region, fresh cerebral suppurations, gynecologic processes, eclampsia, puerperal fever, severe intoxications due to bacterial poisons, heavy metals (lead, sublimate, and etc.), coal gas, Lysol, phenylhydrazine.
7. Subsequent effects of leukopenic infection.
8. Acute exacerbations of chronic infections: tuberculosis and endocarditis.
9. Transition of leukopenic infection into one with severe regenerative complication: influenza with pneumonia.

Neutrophilia with hyperregenerative shift with decreasing or low white blood cell count and absolute aneosinophilia (usually an agonal symptom of very serious significance):

1. Very severe infections.
2. Perforation peritonitis.
3. Lobar pneumonia.
4. Malaria comatosa.
5. Fatal blackwater fever.

Liberation leukocytosis (with involvement of erythrocytes):

1. Complicated bone fractures.
2. Metastases of tumors.
3. Lymphogranulomas.
4. After intravenous injections and transfusions with bad reaction.
5. Blood crises, particularly in pernicious anemia, hemolysis, and blackwater fever.
6. Nonleukemic myelosis (aleukemic myelosis).
7. Marble disease (Albers-Schönberg disease).
8. Simple myelogenous metaplasia of the spleen.

Persisting leukemoid leukocytosis:

1. Persisting direct irritation of bone marrow.
   (a) Carcinomatosis.
   (b) Malignant granuloses.
   (c) Polycythemia vera with hyperplasia of erythropoiesis.
2. Severe anemias complicated with infection.
3. Doubtful acute myelocytic leukemia.
4. Improved stages of agranulocytosis during cure (penicillin).

Neutrophilia with degenerative shift with high normal white blood cell count:

1. Mixed cases (typhoid with sepsis and tuberculosis with secondary infection).

2. Chronic or less virulent infections (hepatic abscess, recurrence, and endocarditis).

Neutropenia with low white blood cell count and marked stab shift:

1. Kala-azar.
2. Other protozoal diseases **after** attacks.
3. During uncomplicated typhoid and paratyphoid fever.
4. Parotitis epidemica in severe cases.
5. Pappataci fever.
6. Brucellosis (this sometimes shows neutrophilia).
7. Influenza.
8. Dengue.
9. Not infrequently in tuberculosis.

Neutropenia with low white blood cell count and no known shift:

1. Pellagra.
2. Yellow fever.
3. Spotted fever.
4. Kedani or tsutsugamushi (scrub typhus).
5. Chickenpox.
6. Poliomyelitis acuta.
7. Ophthalmia sympathica.
8. Pernicious anemia.

Neutropenia with low total white blood cell count and no shift and often total absence of neutrophils:

1. Agranulocytosis.
2. Aplastic anemia.
3. Experimentally in anaphylaxis.

Neutropenia with shift to the right:

Note: These pictures show absence or diminution of stab cells and numerous highly segmented neutrophils with 6 or more segments and dense granules and a tendency to large ovoid cells.

1. Pernicious anemia.
2. Very severe anemias with pernicious blood picture.
    (a) Syphilis.
    (b) Pernicious anemia of pregnancy.
    (c) Sprue.
    (d) Carcinoma of the stomach.
    (e) *Diphyllobothrium latum* infestation.
    (f) Less frequently in other worm diseases.
    (g) Occasionally in malaria and septic processes.
    (h) Less marked in avitaminoses (pellagra).
    (i) Conditions due to insufficient food.
    (j) Cirrhosis of the liver.

Lymphocytosis with increased total white cell count:

1. Constitutional in some individuals.
2. First phase of physiologic or very slight irritation leukocytosis during exercise, digestion, etc.
3. Epilepsy during the attacks.
4. Lymphocytosis of crying nursing infants.
5. Parotitis epidemica during course of mild cases.
6. Pertussis.
7. Uncomplicated benign (afebrile) tuberculosis and syphilitic processes.
8. After certain medicaments such as epinephrine.
9. Lymphocytic leukemia.
10. Acute infectious lymphocytosis.
11. Infectious mononucleosis.

Lymphocytosis with normal or low white blood cell count:

1. All conditions with neutropenia as an associated condition.
2. Lymphocytic aleukemia.
3. Granulomatoses, especially in stages without fever.
4. Neurasthenia and vagotonia (constitutional).
5. Basedow's disease.
6. Acromegaly.
7. Myxedema.
8. Aregenerative anemias, particularly pernicious anemia.
9. Most protozoal diseases during recurrence.
10. During attacks or intervals of conditions caused by viruses.
11. Beriberi.
12. Pure scurvy.
13. Metabolic diseases in chronic stages similar to conditions of hunger.
14. Achylia gastrica simplex.
15. Uncomplicated benign tuberculosis.
16. Uncomplicated syphilis.
17. Continued roentgen exposure.
18. After infections, as a phase of recovery.
19. After vaccinations (typhoid, cholera, dysentery, tuberculin, etc.)

Lymphocytosis with marked low total white blood cell count:

1. Influenza.
2. Pappataci fever.
3. Typhoid fever.
4. Brucellosis.
5. Kala-azar.
6. Agranulocytosis.
7. Aplastic anemia.
8. Pernicious anemia.

Lymphocytopenia with increased white blood cell count:

1. Relative in myelocytic leukemia.
2. Accompanying symptom in conditions with neutrophilia.
3. Severe infections.

Lymphocytopenia with normal, subnormal, or very low white blood cell count:

1. Marked in measles shortly before and after the outbreak of the exanthem.
2. Sometimes in yellow fever and typhus.
3. Malignant attacks of certain diseases with lymphocytic changes (typhoid fever).
4. Paroxysmal hemoglobinuria during attacks.

Monocytosis with increased white blood cell count:

1. In health or slight illnesses if the first drop of blood from the ear is used without cleaning and rubbing the ear.
2. Endocarditis ulcerosa (cells endothelioid and vacuolated).
3. Variola (with the gay blood picture).
4. Typhus in later stages.
5. Measles and German measles.
6. Varicella, less marked.
7. Slight in scarlet fever.
8. Less marked in parotitis epidemica, mild cases.
9. Less marked in acute syphilitic and tuberculous processes.
10. Infections and infectious diseases as a secondary finding with good prognosis.

11. Monocytic leukemia.
12. Malignant endocarditis lenta ulcerosa (macrophages).

Monocytosis with low normal to high normal count of white blood cells:

1. Chronic vascular diseases, such as severe arteriosclerosis, in recovery, especially near the crisis.
2. Chronic syphilis and tuberculosis.
3. Slight in malignant tumors.
4. Acute protozoal diseases during the attacks (malaria and trypanosomiasis).
5. Severe cases of parotitis epidemica.
6. Basedow fruste.
7. Helminthiasis.
8. Hyporegenerative septic processes as a secondary finding.
9. Agranulocytosis.
10. Banti's symptom complex.
11. Measles.
12. Yellow fever.
13. Dengue fever.
14. Spotted fever.
15. Ophthalmia sympathica.
16. Poliomyelitis.
17. Pellagra.

Decrease or absence of monocytes:

1. Very severe infections as a very unfavorable symptom.
2. Myelocytic and lymphocytic leukemias.

Chapter 66

Myeloproliferative disorders

JOHN D. BAUER

In the group of myeloproliferative disorders[721] a number of diseases are collected which may or may not be related and which at times overlap but which have the following features in common: their precise etiology is unknown, they originate in the bone marrow or other erythropoietic sites, and they are characterized by the proliferation of one or the other or of all elements which occur in the bone marrow.

In many of the previous chapters benign, reversible, or reactive bone marrow proliferation following a known stimulus such as blood loss, infection, etc., has been described. In the myeloproliferative disorders the cause is unknown, and the proliferation is progressive, irreversible, and thus malignant. The borderline of benign hyperplasia is by no means clearcut, and it is at times difficult or impossible to decide at a given time whether a proliferative process is benign or malignant.

CLASSIFICATION

- Chronic types
  - Polycythemia vera
  - Myelosclerosis with myeloid metaplasia
  - Chronic granulocytic leukemia
  - Chronic erythemic myelosis (Heilmeyer)
  - Aplastic anemia (chronic congenital hypoplastic anemia)
- Acute types
  - Acute erythremic myelosis (Di Guglielmo's disease, erythremia, erythroleukemia)
  - Acute granulocytic leukemia
  - Acute megakaryocytic leukemia
  - Acute myelosclerosis with myeloid metaplasia
  - Thrombocythemia
  - Aplastic anemia

Some of these disorders, as stated previously, are poorly delineated, poorly understood, overlap, and fade into each other so that mixed forms occur—erythromegakaryocytic myelosis, erythroleukemic myelosis, etc.

Some of the conditions classified as myeloproliferative disorders, such as aplastic anemia, myelosclerosis, and myelofibrosis, produce a clinical condition referred to as bone marrow failure, relative bone marrow failure, refractory anemia, or aplastic anemia. In some of these conditions the bone marrow morphology fails to correlate with the peripheral blood picture.

Under the heading of chronic bone marrow failure the following conditions should be mentioned: pure red cell anemia (chronic simple anemia), myelophthisic anemia, and idiopathic bone marrow failure.

APLASTIC ANEMIA

The inclusion of aplastic anemia in the myeloproliferative disorders may at first seem strange until one realizes that aplasia and hyperplasia are frequently close neighbors. The stimulated hyperplastic bone marrow in benzol poisoning may become exhausted and give way to a bone marrow devoid of blood-forming elements, whereas the hypoplastic bone marrow of acute leukemia may blossom into the hypercellular stage of fully developed leukemia.

Aplastic anemia is usually regarded as a well-defined, readily recognizable clinical entity, with features that are the result of a progressive depression and final cessation of bone marrow activity and aplasia of this tissue. Ordinarily, one does not think of regeneration in these cases, but Thompson et al.[722] reported 13 cases of aplastic anemia in which there was evidence of blood regeneration. The first case of aplastic anemia on record is that by Ehrlich.[723] In this case the red cell count was 213,000, no white cell count was reported, no blood platelets were reported, and the tibial bone marrow was yellow and fatty. By 1908 Cabot[724] had collected 24 cases, and Hirschfeld[725] in 1911 found the total to be 44. Musser[726] reviewed the situation up to 1914. In 1919, Smith collected 64 cases. He found that it was a disease of young adults that progressed rapidly and was characterized by a progressive decrease in the number of formed elements in the blood. Root[727] and Carey and Taylor collected, in 1931, 150 cases.

It is accepted that idiopathic aplastic anemia is a uniformly acute, rapidly fatal disease of adolescents and young adults, with a high fever, a rapid decrease in the cells of the blood with very little suggestion of regeneration of the blood cells. At autopsy the bone marrow shows complete fatty degeneration.

The blood picture is a lowered count, often below 2,000,000, and a marked lowering of hemoglobin. Although there is a rapid decline in the number of red cells, the form of the cells is not markedly changed. It is significant that there are no nucleated red cells and very little polychromasia. Reticulocytes are very scarce. The white cell count declines, with a decline of the neutrophils and disappearance of eosinophils, and relative lymphocytosis supervenes. There is marked leukopenia in advanced cases of this disease. The final picture, so far as leukocytes are concerned, is practically all lymphocytes. Another very characteristic phenomenon in the blood picture is the decline and absence of blood platelets. It is significant that when the blood platelets drop very low purpuric symptoms occur.

From a study of the literature, it seems that there are 3 variants of aplastic anemia. There are cases in which the disease is acute in its course, without evidence of regeneration and with complete aplasia of the bone marrow at autopsy. In addition, there is a second group comprising a few cases with clinical features similar to the preceding group, but in which the marrow, instead of being fatty, has a normal or even increased cellular content. Such cases have been reported by Naegeli,[728] Senator,[729] Wolff,[730] and Root[727] and several appear in the Italian literature[731-733] under the name of pseudoplastic anemia, a term suggested by Luzzatto[733] to indicate the discrepancy between the blood and bone marrow pictures. The third variation includes progressive hypocythemia cases in which evidence of regeneration appeared in the peripheral blood.

According to Thompson et al., examination of the peripheral blood may be no index as to the morphologic appearance of bone marrow examined, and the morphologic appearance of the bone marrow may be no index as to its functional activity. There may be quite serious interference with the normal course of development, maturation, and division of blood cells without alteration in the cellular content of the marrow.

Krumbhaar in commenting upon the case reports and conclusions of Thompson et al. stated very properly that these conditions should be described by 3 different labels as follows: aplastic anemia to be reserved for the type without evidence of regeneration in blood or bone marrow, pseudoaplastic anemia for the variety with cellular bone marrow, and progressive hypocythemia for the cases corresponding to those of Thompson et al. in which there is an evidence of attempted regeneration in both bone marrow and blood.

In summary we then distinguished the following types of aplastic anemia: primary aplastic anemia and secondary aplastic anemia.

Primary aplastic anemias

Pure aplastic anemia (pure red cell anemia): This anemia is characterized by a progressive normocytic normochromic anemia. The bone marow shows disappearance of erythroblasts and normoblasts. In the peripheral blood no evidence of red blood cell regeneration can be found, but the complement of platelets and white blood cells is essentially normal. There is no enlargement of the spleen, lymph nodes, or liver. Such anemias have been described in association with thymomas and acute renal failure and following nerve resection.

Pseudoaplastic anemia (hypoplastic anemia): The bone marrow may be cellular or even hypercellular, but because of a maturation arrest at the normoblastic level, the peripheral blood lacks reticulocytes. Splenomegaly and hepatomegaly are absent. This anemia also goes under the name of refractory anemia.

Hypoplastic anemia of Diamond-Blackfan (congenital): This anemia is characterized by a hypoplastic marrow erythropoiesis rather than by complete aplasia. It is found in newborn infants and children. It is at times associated with other congenital defects such as Fanconi's syndrome. It is interesting to speculate that a myeloid-lymphatic antagonism is etiologically involved in the production of this anemia. The existence of such an antagonism can be postulated in the pure red cell anemia associated with thymomas. In children who characteristically show hyperplasia of lymphatic tissues, a similar mechanism could be considered responsible for the production of the bone marrow depression. The hematologic findings vary from a macrocytic to a normocytic anemia, without reticulocytosis or only a minimal increase in reticulocytes.

Idiopathic aplastic anemia: This anemia shows depression of all blood-forming marrow elements: erythroblasts, granulocytic elements, and megakaryocytes. The blood picture reflects the results of marrow failure.

Secondary aplastic anemias

Secondary aplastic anemias may follow irradiation therapy or the administration of cytotoxic drugs or drugs to which an idiosyncrasy may develop.

One of the most important drugs that selectively suppresses the bone marrow erythropoiesis is chloramphenicol.[734, 735] In patients who are sensitive to this drug within 48 hr. after administration of chloramphenicol vacuolization of the erythroblastic cytoplasm appears, and the peripheral reticulocytes fall below 0.5%. If the drug is not discontinued, irreversible aplastic anemia may result.

Hematologic findings

The red blood cells show a severe normocytic normochromic anemia. The polymorphonuclear cells are at times slightly increased and may show toxic granulation. Usually there is an increase in lymphocytes and monocytes. Thrombocytopenia is usually present. The bone marrow shows hypoplasia of all elements, and if erythroid elements are seen, they may be abnormal. The megakaryocytes may be decreased or increased in number.

Nonhematologic findings in aplastic anemia

The nonhematologic findings include an elevated serum iron and an abnormal amino acid excretion pattern of the urine.

Etiology

The actual cause is unknown. The most interesting aspect of some of the aplastic anemias is the hormonal dependence exhibited by their response to therapy with steroids and testosterone.[736, 737]

Differential diagnosis

Aleukemic leukemia associated with hypocellular bone marrow may mimic aplastic anemia so closely as to be indistinguishable until immature cells are found in the peripheral blood. Bone marrow aspiration or surgical biopsy (Vim-Silverman needle) may be helpful in the differential diagnosis.

Myelophthisic anemia: The anemia is the result of replacement of the bone marrow by nonhemopoietic tissue, such as fibrous tissue (myelofibrosis, myelosclerosis), by tumors such as lymphomas (Hodgkin's disease), and by metastases of carcinoma the primary site of which is usually the lung, thyroid, breast, or prostate. The bone marrow space may also be occupied by granulomas such as tuberculosis or by reticuloendothelioses. The hemopoietic activity is taken over by the spleen and liver which develop extramedullary hematopoietic activity.

Myelofibrosis: This is an obliterative bone marrow disease of many years' duration. The liver and spleen enlarge markedly due to the tremendous hemopoietic activity (agnogenic? myeloid metaplasia). The peripheral blood picture is characterized by a severe anemia exhibiting anisocytosis, poikilocytosis (teardrop cells), and normoblastosis. Not only is there leukocytosis, but also immature white cells are swept into the blood stream, producing a picture not unlike that of chronic granulocytic leukemia. The increase in the number of platelets adds to the difficulty in distinguishing myelofibrosis from myelogenous leukemia, which at times is accompanied by marrow fibrosis. The differential diagnostic points are described in the discussion of chronic myelogenous leukemia. In addition to the hematologic consideration just mentioned, bone biopsy (needle or surgical) and radiographic examination of the bones may be helpful.

Marble bone disease (Albers-Schönberg): This disease, which begins in childhood, is characterized by replacement and finally obliteration of the bone marrow cavities by bone. The anemia is of the leukoerythroblastic type, similar to the one in myelosclerosis. The liver and spleen enlarge as they take on hematopoietic function. Diagnosis is based on the leukoerythroblastic anemia, bone marrow biopsy, and characteristic radiologic findings.

POLYCYTHEMIA (ERYTHROCYTOSIS AND ERYTHREMIA)

Table 268 shows the nomenclature prepared by the American Society of Clinical Pathologists' Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-Forming Organs.

Table 268. *The erythrocytoses**

Term to be used	*Terms to be avoided*
Erythrocytosis due to polycythemia vera	Erythremia, polycythemia, Vaquez's or Osler's disease, erythremia, splenomegalic polycythemia, Gaisböck's disease, polycythemia hypertonica, erythroleukemia, polycythemia rubra vera, erythrocytosis megalosplenica
Arterial anoxemia due to:	Erythrocytosis, erythremia, erythroleukosis
Unknown cause	
Heart disease	
Pulmonary disease	
Arteriolosclerosis in lungs	Ayerza's disease
Hereditary leptocytosis (trait)	

*From Am. J. Clin. Path. **20**:562, 1950.

Classification

Polycythemia is a term used to describe an increase in the number of circulating erythrocytes. Several types of polycythemia can be distinguished.

Relative polycythemia—synonymous with hemoconcentration produced by plasma loss leading to an increase in the red blood cells per unit volume (dehydration).

Secondary polycythemia (erythrocytosis)—characterized by in increase in red blood cells due to increased erythropoiesis secondary to chronic anoxemia.

Polycythemia vera or erythremia—a disease of unknown etiology characterized by increased production of red blood cells, white blood cells, and platelets.

Symptomatic polycythemia—erythrocytosis accompanying various primary diseases not associated with any form of anoxemia as far as it is known.

Relative polycythemia

Relative polycythemia may be caused by dehydration and may give the patient the appearance of absolute polycythemia. In this condition, however, there is a diminished blood volume but no real overproduction of red blood cells. This occurs following excessive exercise in which there has been an unusual excretion of water through the skin and lungs. Prolonged vomiting, diarrhea, and fever may be causative factors. In other words, it is due to loss of water from the blood plasma following abnormally lowered fluid intake or marked loss of body fluids (dehydration). The differential diagnosis depends upon the presence of increased red blood cells associated with diminished blood plasma volume as determined by the hematocrit. The condition is usually transitory, definite improvement occurring by dilution of the blood with fluids.

Secondary polycythemia or erythrocytosis

Secondary polycythemia or erythrocytosis usually represents an effort on the part of the organism to compensate for difficulties in the oxygenation of the blood or tissues of the body, the underlying cause of the increased blood production being oxygen want. First, there may be defective saturation of arterial blood with oxygen as evidenced by the erythrocytosis of high altitude (mountain sickness). This occurs not only in people who live in high altitudes but also in travelers who go up in planes or climb mountains. Blood counts may reach 7,500,000 or 8,000,000 cells/mm. This condition from a similar cause (that is, poor oxygenation) is also found in newborn infants, congenital heart disease, and certain forms of acquired heart disease and pulmonary diseases and in those conditions due to a defect in the hemoglobin of the blood so that it is unable to combine with oxygen in normal amount as manifested by the toxic action of various bacterial and chemical poisons on the red blood cells.

In this classification are thus such conditions as mountain sickness, chronic heart disease, cyanotic congenital heart disease, *e.g.* tetralogy of Fallot associated with shunt of venous blood into the arterial system prior to aeration, pulmonary emphysema, pulmonary fibrosis, pulmonary hypertension, pulmonary arteriovenous fistula, pulmonary artery sclerosis, certain blood diseases such as methemoglobinemia, hemolytic anemias, chronic carbon monoxide poisoning, and early stages of others such as myelosclerosis, leukemia, etc. It might be mentioned here that the recovery stage of some anemias may be associated with transitory polycythemia.

In secondary polycythemia red cell counts as high as 13,900,000 have been recorded and hemoglobin as high as 23.7 gm. (normal, 13-15 gm.), whereas the volume of packed red cells averages 54.4/100 ml. (normal, 41.9-42-9 ml.).

Polycythemia vera or erythremia

Polycythemia vera or erythremia is a slowly, intermittently progressive disease of unknown etiology characterized by a striking absolute increase in the number of red blood corpuscles and the total blood volume and frequently by signs of generally increased bone marrow activity.

Attention was called to this disease in 1892 by Vaquez some time following the development of a method for counting red blood corpuscles in the middle of the nineteenth century.

The disease is more common in males than females; a fair percentage of the cases are found in Jews born in Eastern Europe. It usually occurs in middle or late life. Several persons of the same family may be affected. The majority of workers in this field believe that the pathologic physiology is an increased activity of the blood-forming apparatus.

Patients are usually vague in their statements concerning the date of onset of symptoms. From histories of slight discomfort or vague ill-defined complaints it is evident that the disease is likely to have been present for a long time before it finally obtruded itself upon the consciousness of the patient. Individuals may apply to the physician for cosmetic reasons to be rid of the unnatural red flush of the skin and may be otherwise symptom free. Such general symptoms as weakness, lassitude, easy fatigability, loss of weight, and shortness of breath are frequently present. Various gastrointestinal symptoms including abdominal pain, eructations of gas, anorexia, nausea, and vomiting may occur. Hemorrhages into the skin and mucous membranes are not uncommon. Nervous symptoms manifested by headache, dizziness, noises in the ears, tingling sensations of the hands and feet, staggering gait, muscular weakness, blurring of vision, thickness of speech, and even loss of consciousness may be complained of.

On physical examination one finds the characteristic reddish blue color of the skin and mucous membranes, or there may be a definite pallor. The spleen is enlarged in the majority of patients; the size of the spleen varies greatly at different periods of the disease. Enlargement of the liver occurs in more than 50% of the patients. Blood pressure is usually within normal limits. Muscular twitchings or paralysis may be present. There is no enlargement of the heart in the majority of the cases. Palpitation is complained of by many patients. The disease may be latent for years. After it has become fully manifest, its course is chronic, slowly progressive, and ultimately fatal. Patients may live from 4-20 yr. or more after the onset of the recognized symptoms. Death usually results from vascular complications, especially cerebral thrombosis or hemorrhages. Serious and often fatal hemorrhages have occurred from esophageal varices or from blood vessels of the stomach, lungs, or other viscera. Heart failure may be the terminal event.

Examination of the blood reveals a marked increase in the red cell count. It usually ranges from 7,000,000-12,000,000/c.mm., the highest authentic count recorded being 13,600,000/c.mm. It has been calculated theoretically that with red cells of normal size the circulatory system would be unable to contain the blood if the count exceeded 13,900,000/c.mm. The blood is characteristically dark and spreads slowly under the cover glass. It may be so thick that it is drawn up into a pipet with difficulty. Patients with 80 ml. packed red cells/100 ml. blood have been observed.

In polycythemia the red blood cells are often normal in size, but it is not uncommon to find that their mean volume is less than normal. Thus the average size in 14 cases was 83 cμ—the smallest cells being about 61 cμ.

The hemoglobin is also increased but less than the red cells. Hemoglobin values varying from 18-24 gm.% are common.

The blood counts vary appreciably from day to day; spontaneous remissions and relapses occur. Simultaneous blood counts from venous and arterial blood show in general no marked differences.

The red blood cells as examined in the blood spread may appear quite normal; however it is not unusual to find somewhat greater variation in size than is normal and more rarely, poikilocytosis. Microcytes are not infrequent; macrocytes are unusual. Especially when the disease has become more advanced and particularly when hemorrhage has occurred, the red cells appear paler than normal. Polychromatophilia and less frequently basophilic stippling may be encountered. Nucleated red cells are not rare, but other indices of increased erythrogenesis are almost never encountered. Anisocytosis, poikilocytosis, polychromatophilia, and normoblastosis may be quite marked. Special staining reveals frequently a slightly increased reticulocyte count, often about 1-2%. Increases in reticulocytes are found following hemorrhages only.

Leukocytosis is quite common, the count varying from 10,000-25,000, but values up to 50,000/c.mm. are by no means rare. The leukocytosis is due to an absolute increase in the number of granulocytes. The cells of the myeloid series comprise 70-95% of the leukocytes. Metamyelocytes are found in increased number, and it is not unusual to find 1-2% myelocytes; myeloblasts have also been observed. The eosinophilic and basophilic leukocytes may be increased although this is less common. Lymphocytes are relatively reduced, but usually their absolute number is about normal. Monocytes may be absolutely increased.

The blood platelets are usually increased; counts of 1,000,000/c.mm. are quite usual, and in some cases the count may exceed 3,000,000. Megakaryocytes are rarely found in the circulating blood.

Bleeding time is usually essentially normal, and coagulation time is normal. Clot retraction may be slow. The poor clot retraction has been related to the increased number of platelets, but recently Abraham et al.[738] found evidence of a platelet factor 3 deficiency. Disturbances in fibrinogen contents and deficiencies of factor V and factor VII have been reported.

Fragility of the red cells may be normal but is frequently increased. In dilutions of their own or normal serum, the patient's cells might yield an abnormally long resistance span. Normal cells however may be hemolyzed in dilutions of the patient's serum.

The viscosity of the blood in polycythemia is much greater than that in normal blood. It may be 5-8 times greater. This is due primarily to the great excess of red blood cells over plasma. The viscosity of the serum, indeed, is said to be less than is normally found in health. Likewise the specific gravity of erythremic blood is 1.075 to 1.080 as compared with 1.055 to 1.065 for normal blood. The sedimentation rate of polycythemic blood is very greatly delayed.

The total volume of blood in erythremia as measured by both the dye and carbon monoxide methods is found to be greatly augmented. Volumes of 121-246 ml./kg. body weight have been found as compared with a normal value of 87 ml. Plasma volumes are increased little or not at all. There is an increase in the blood in the organs in general, and even the smaller vessels of the pia mater of the brain and cord are engorged and distended with blood.

Sternal bone marrow puncture reveals a dark red marrow crowded with enormous numbers of mature erythrocytes as well as with normoblasts. All the nucleated red cells are small and contain round, ripe, often pyknotic nuclei. Proerythroblasts are frequently few or absent. Polychromatophilic red cells are few. The leukopoietic apparatus is also overactive, with myelocytes and metamyelocytes predominating; even the promyelocytes may be increased in number. Mature neutrophils are reduced in number. Megakaryocytes are frequently increased.

Complications: The most important complications are vascular in type. There is frequent (about 30%) occurrence of thrombosis, which may involve the extremities, the cerebrum, the heart, or of course other sites. About 10% of all patients develop peptic ulcers which may well be a thrombotic phenomenon. The other vascular phenomenon is hemorrhage. The slight coagulation defect coupled with congestion and distention of the vascular bed leads to hemorrhage, mainly from the mucosa of the gastrointestinal tract.

Gout is a somewhat infrequent complication though moderate hyperuricemia is commonly found. The latter is more marked if myelosclerosis and myeloid metaplasia are associated with polycythemia.

The ultimate fate of some of these patients is responsible for the inclusion of polycythemia vera among the myeloproliferative syndromes. It is stated that 20-60% of the patients with polycythemia, if they survive a period of approximately 10 yr., will die with signs and symptoms of chronic granulocytic leukemia or erythroleukemia. About 30% of these patients develop myelosclerosis characterized by an enlarged spleen and immature red and white cells in the peripheral blood (myeloid metaplasia). These terminal changes are not the result of treatment with P^{32} or radiation since they were seen before the advent of P^{32}. A further proof of some pathophysiologic relationship between polycythemia, leukemia, and myelosclerosis lies in the fact that primary myelosclerosis may initially show a polycythemic blood picture. A few cases of pernicious anemia have been reported which terminated as polycythemia vera. In some of the cases reported as granulocytic leukemia following polycythemia vera, the alkaline phosphatase content of the white cells was not decreased as is expected in granulocytic leukemia but rather was elevated. Some of the cases reported as myelogenous leukemia may thus in fact represent examples of extreme granulocytic hyperplasia. There is evidence that elevation of the serum lactic acid dehydrogenase will foretell the leukemic termination of polycythemia vera.

Ferrokinetics of polycythemia vera: There is an increased plasma iron turnover. The iron disappears rapidly from the plasma and is rapidly incorporated into the red cells. The daily hemoglobin synthesis in polycythemia vera is several times the normal rate.

Pathogenesis: As stated, the cause of polycythemia vera is unknown. Neither anoxemia, central stimulation, nor overproduction of erythropoietin can be accepted as the etiology. The many reported cases of polycythemia associated with renal disease proved, on detailed examination, to be instances of erythrocytosis rather than polycythemia, and are included in the discussion of erythrocytosis. The final leukemic stage of polycythemia brings to mind Dameshek's concept of an "acquired self-perpetuating metabolic defect due to enzyme deletion," which he considered to be an etiologic possibility in Di Guglielmo's disease.

Treatment of polycythemia: Dameshek[739] gave an excellent summary of the present concept concerning the treatment of polycythemia.

The treatment of polycythemia has passed through several successive stages since Osler first used benzol to reduce the excessive blood cell formation. Phenylhydrazine, a hemolytic poison, was introduced by Morawitz following its experimental use by J. H. Pratt. Although this new drug achieved wide popularity, its unpredictable nature and the severe hemolytic crises which often ensued made its use relatively hazardous.

In Dameshek's experience, the removal of 500 ml. blood twice weekly for 2-5 wk., depending upon the initial hematocrit and hemoglobin levels, has proved a satisfactory method. To maintain somewhat longer the resulting state of iron deficiency, it has been his practice to keep the patient on a diet low in iron. Red cell formation under these circumstances is only partially reduced, but hemoglobin and hematocrit levels remain low for periods of 6-18 mo., during which time the patient may be completely asymptomatic. Red cell levels during this induced remission gradually rise so that the red cell count as an index of therapy is of little value. The best index is the hematocrit value although the hemoglobin concentration alone may be used since this correlates fairly closely with the hematocrit level. With this method of therapy patients go along for many years with very little more difficulty than do others in the older age group in which polycythemia occurs.

The use of x-ray therapy has in Dameshek's hands proved ineffective, either by heavy dosage to localized areas or by spray or generalized therapy. He has hesitated to use high voltage x-ray in these essentially normal individuals because of the possible dangers of radiation malignancy or leukemia. The introduction of radioactive phosphorus, as discussed by Erf,[740] makes therapy a good deal easier and is ordinarily productive of an excellent remission.

Symptomatic polycythemia

In symptomatic polycythemia the relationship between red cell volume and plasma volume remains normal. There is no splenic tumor.

The etiology of many of these erythrocytoses is unknown, and they usually show only a rise in the red blood cells.

Polycythemia of stress: Dameshek[741] described this well-accepted entity in 1953. These individuals are usually men of the plethoric, obese, aggressive type with elevation of blood pressure. Acrocyanosis of the hands and feet may be present. Other than an elevation of the red blood count and hematocrit, all hematologic data are normal. Arterial oxygen saturation is normal, bone marrow studies are normal, and the red blood cell volume is normal. Plasma volume, however, is low. Lawrence and Berlin[742] emphasize the low plasma volume and suggest that it may be the result of long-continued psychic stress. These patients have such disturbances as peptic ulcer, coronary disease, and hypertension, a triad familiarly associated with psychosomatic disturbances. Dameshek stresses recognition of this entity, chiefly to avoid the type of vigorous therapy traditionally given patients with polycythemia vera.

Erythrocytosis associated with tumors: The following tumors have been seen associated with polycythemia: cerebellar hemangioblastoma, hypernephroma, uterine fibroids,[743] and cardiac myxoma. Pathophysiologic pathways may include erythropoietin production, intravascular erythropoiesis, or diencephalic stimulation.[744]

Erythrocytosis and kidney diseases[745]: Apart from the aforementioned renal cell carcinomas, erythrocytosis has been linked to hydronephrosis, renal cysts, adenomas, metastatic tumors to the kidney, and renal tuberculosis. It appears most attractive to make the increased production of erythropoietin responsible for the erythrocytosis.

Erythrocytosis and endocrinopathies[746]: Cushing's syndrome is usually accompanied by an increase in circulating red blood cells. If one accepts the hypothalamic etiology of Cushing's syndrome, the erythrocytosis may be classified as cerebral in origin, similar to the one seen in some cases of encephalitis or brain tumors. If the spleen can be classified as a hormone-producing organ, the increase in red cells accompanying splenic tuberculosis or hyposplenism may fall into this category.

Laboratory diagnosis of polycythemias

The differential diagnosis[747] of the polycythemias is presented in Table 269.

Bioassay of erythropoietin[748]: Methods to measure erythropoietin utilize live rats which are rendered sensitive to the action of exogenous erythropoietin contained in the assay material, such as human plasma, serum or kidney tumor extracts, kidney cyst fluid. The endogenous erythropoietin of the rat is suppressed by various procedures such as starvation, transfusion polycythemia, hypophysectomy, and others. Fe^{59} is used to measure changes in red cell production.

DI GUGLIELMO'S SYNDROME

This disease is among the many myeloproliferative disorders. It represents a primary disease of the erythropoietic bone marrow. It is divided into 2 groups: acute erythremia (Di Guglielmo's disease) and chronic erythremia (Heilmeyer-Schöner disease).

Acute erythremia (Di Guglielmo's disease)

Clinically, this disease is characterized by an acute onset, by severe anemia, and by hemorrhagic and hemolytic manifestations.

Hematologic findings:

Red blood cells: There is a severe progressive normochromic or hypochromic hemolytic anemia characterized by a diminished number of red blood cells, associated with a low hematocrit and hemoglobin values. The peripheral blood film shows anisocytosis, poikilocytosis, and macrocytosis. Numerous erythroblasts are seen, mainly basophilic early forms and relatively few more mature erythroblasts. Many of the erythroblasts are pathologic and show irregular lobulated nuclei, with evidence of abnormal nuclear cytoplasmic maturation and ratio.

White blood cells: The white blood cell count is normal to diminished or slightly increased.

Platelets: Platelets are always diminished.

Bone marrow: Most normal bone marrow elements are crowded out by erythroblastic hyperplasia characterized by predominance of basophilic erythroblasts over the more mature erythroblasts. Megaloblasts and giant erythroblasts are seen as well as pathologic multinucleated and multilobulated erythroblastic forms.

Nonhematologic findings:

Plasma: The plasma vitamin B_{12} level

Table 269. *Laboratory diagnosis of polycythemias*

Laboratory test	*Relative polycythemia (hemoconcentration)*	*Erythrocytosis (secondary polycythemia)*	*Erythremia (polycythemia vera)*
Number of red blood cells	Increased	Increased	Increased
Immature red blood cells	None	None	Occasionally
Number of white blood cells	Increased	Normal	Increased
Hemoglobin	Increased	Increased	Increased
Hematocrit	Increased	Increased	Increased
Red cell mass	Normal	Normal	Increased
Plasma mass	Decreased	Normal or decreased	Normal or decreased
Number of platelets	Increased	Normal	Increased
Oxyhemoglobin saturation	Normal	Decreased	Normal
Uric acid	Normal	May be increased	Increased
Bone marrow	Normal	Erythroid hyperplasia	Hyperplasia of all hematopoietic elements
Basal metabolism (apart from thyroid dysfunction)	Normal	Normal	Increased
Sedimentation rate	Increased	Normal	Increased
Plasma erythropoietin	Normal	Normal	Increased
Serum iron	Normal	Increased	Decreased
Plasma iron clearance and turnover	Normal	Normal	Accelerated

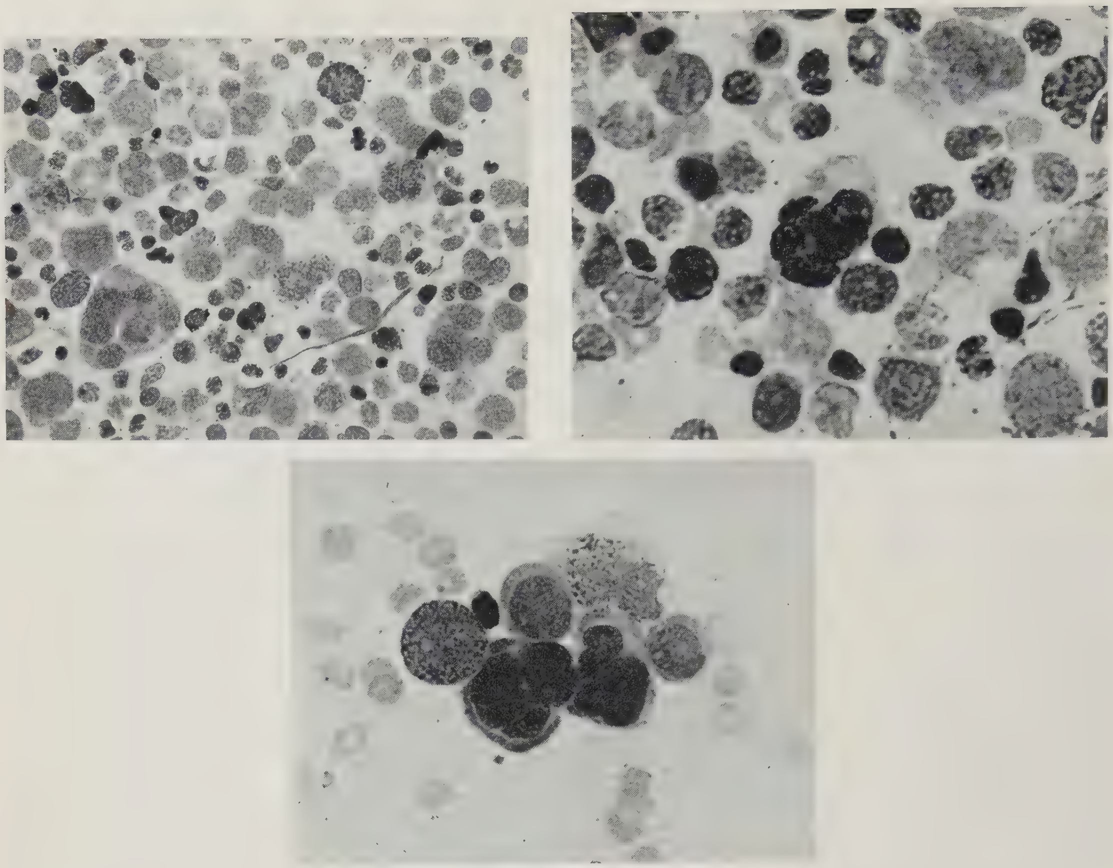


Fig. 440. Bone marrow in erythroleukemia. Giant multinucleated bizarre pathologic erythroblasts and giant multinucleated normoblasts.

is elevated, and there is evidence of hemolysis.

Pathology: Di Guglielmo states that the main pathologic disorder lies in the erythropoietic system which shows hyperplasia (increase in number of erythroblasts) accompanied by anaplasia of the erythroblasts (inability to mature) and dysplasia (nuclear and cytoplasmic abnormalities). For a short discussion of etiologic concepts see the final paragraph of the section on pernicious anemia.

Course of disease: The disease is a myeloproliferative disorder, and true to form as it progresses, evidences of acute leukemia may appear (erythroleukemic form). Finally the picture may become one of pure acute myelogenous leukemia.

Chronic erythremia (Heilmeyer-Schöner disease)

This disease is similar to the acute form though clinically it is of longer duration and is less aggressive. Hematologically it is more dysplastic than anaplastic (the words dysplasia and anaplasia are used in Di Guglielmo's sense, see discussion of acute erythremia).

Mixed forms

In the evolution and course of acute erythremia various mixed forms occur. These conditions are self-explanatory and were named by Di Guglielmo as follows: erythroleukemia, erythromegakaryocytic leukemia, and erythroleukomegakaryocytic leukemia.

The morphologic features of these diseases are by no means clear-cut, and the characteristics of each overlap to such an extent that it is at times difficult to establish a definitive diagnosis. Not only is there some difficulty in delineating these subgroups, but it is also at times difficult to establish the diagnosis of Di Guglielmo's disease. The differential diagnosis includes not only acute and chronic leukemia with symptomatic erythroid hyperplasia, but, more important, also myelosclerosis, severe hemolytic anemias, polycythemia, and pernicious anemia. Such laboratory procedures as serum iron determination, stain for alkaline phosphatase in polymorphonuclear cells, serum level of vitamin B_{12}, and surgical biopsy of bone marrow may all be helpful in the differential diagnosis.

LEUKEMIA

The historical development of leukemia might be said to begin with a description of the disease by Virchow in the eighteen fifties, when he outlined the "lienal" and "lymphatic" forms of leukemia. Between 1875 and 1877

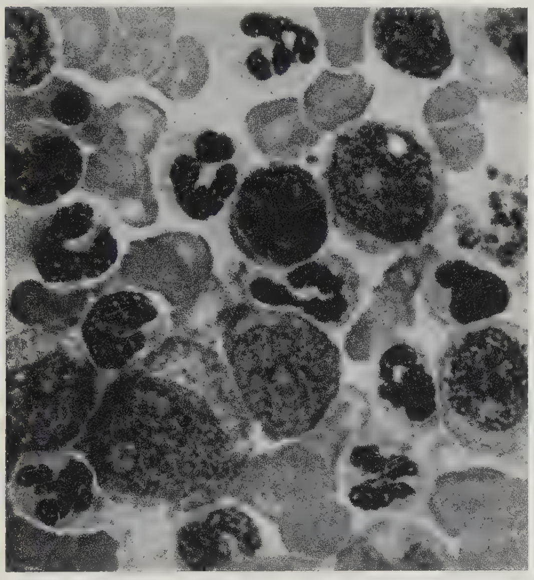


Fig. 441. Myelocytic leukemia, blood film, showing crowding of cells and increase in large myelocytes and myeloblasts. (×950.)

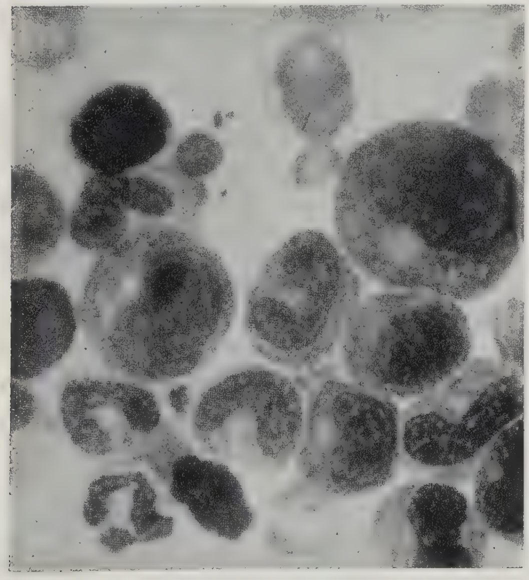


Fig. 442. Blood film, leukopoiesis, showing large number of myelocytes and juvenile cells. (×950.)

Table 270. *Leukemias and lymphomas**

Term to be used	*Terms to be avoided*
Leukemia and lymphoma generally	
Leukemia	Leukosis, leukanemia, leukosarcoma, leukocythemia
Type unclassified	
Lymphocytic	
Acute	Lymphoblastic leukemia or blast cell leukemia, lymphadenosis, lymphocythemia, lymphatic, lymphoid or lymphogenous leukemia
Leukemic	
Subleukemic	
Aleukemic	
Subacute	
Leukemic	
Subleukemic	
Aleukemic	
Chronic	
Leukemic	
Subleukemic	
Aleukemic	
Granulocytic	Myeloblastic leukemia
Neutrophilic	Myeloid, myelogenous, myelocytic leukemia, myelosis, myelemia, myelocythemia
Eosinophilic	
Basophilic	
Monocytic	Reticuloendotheliosis, histiomonocytic, Schilling type, myelomonocytic, or Naegeli type leukemia
Plasmocytic	
Megakaryocytic	
Lymphoma	Lymphoblastoma, lymphocytoma, malignant lymphadenoma
Type unclassified	
Lymphocytic sarcoma	Lymphosarcoma
Reticulum cell sarcoma	Reticulosarcoma
Follicular lymphoma	Giant follicular lymphoma, giant follicular lymphoblastoma, lymphoid follicular reticulocytosis, Brill-Symmer disease
Hodgkin's disease, unspecified	Hodgkin's lymphoma, Hodgkin's lymphoblastoma, Hodgkin's pseudoleukemia lymphogranulomatosis
Hodgkin's paragranuloma	
Hodgkin's granuloma	
Hodgkin's sarcoma	
Mycosis fungoides	
Spiegler-Fendt sarcoid	
Plasmocytic myeloma	Multiple myeloma, plasma cell myeloma, plasmocytoma, myelocytoma, Kahler's disease
Erythrocytic sarcoma	Erythroblastoma
Mixed types	

*From Am. J. Clin. Path. **20**:571, 1950.

Virchow differentiated leukemic cell increase from the leukocytoses due to pus formation. With the advent of selective stains into hematology there began recognition of the difference between the lymphocytic and myelocytic leukemias. Then came Naegeli's characterization of the myeloblast which gave this dualistic conception of the disease a firm basis. With the final appearance of the oxydase reaction, it was possible to differentiate myelocytic from lymphocytic cells histologically.

What is leukemia? Leukemia has many features of a malignant neoplasm. The progressive, purposeless growth of the leukemic cells, leading to the death of the patient, is good evidence for the autonomous nature of this disease. In animals, as in man, leukemia may be but one phase of tumor growth. Several years ago (1953) Custer showed the interrelationship of lymphosarcoma and Hodgkin's disease on the one hand and lymphatic and monocytic leukemia on the other hand. The endothelioma of fowl, after many passages, becomes leukemia, and fowl leukemia after many passages will become sarcoma.

Leukemia or at least some forms of it may be of a nonneoplastic nature. The evidence is meager. Leukemic cells fail to grow in the anterior chamber of the rabbit eye, which allows growth of most other human tumors. This behavior may be related to some specific environment these cells require.

Leukemia may be a viral disease.[749] Animal experimentation strongly favors this view. Electron microscopic pictures of chicken leukemic cells reveal viruslike particles. In animals leukemia can be transferred by means of cell-free filtrates from a high leukemic strain to a low leukemic strain. Viruslike inclusions have been reported in human leukemias. Injection of urine of leukemic patients into high leukemic mice strains will give rise to leukemia.

Ellermann has described a chicken leukemia which may be transferred by means of a filtrable virus but which alternately shows myelocytic, lymphocytic, and erythropoietic hyperplasias. Snijders[750] reported, in 1926, a large cellular leukemia of a guinea pig. One hundred forty-eight other guinea pigs which were inoculated with living cells from the first pig developed leukemia; some showed tumorlike infiltrations, some highly tumorous leukosarcomatosis, etc. The proliferating cells all seemed to originate from the transplanted foreign cells.

Leukemogenic factors:

Heredity: Some strains of mice (AKR mouse) show a high incidence of leukemia, whereas others rarely develop leukemia. In human beings and in higher animals there are reports of familial predisposition.[753] A genetic factor[754] is also suggested by the association of acute leukemia and mongolism, a disease which shows an abnormal chromosomal pattern.

Exposure to radiation[755]*:* Two separate problems must be considered—the short more or less single exposure to large amounts of irradiation and the repeated exposure to small doses of radiation. Hiroshima and Nagasaki provided the answer to the problem of massive exposure. The problem of the leukemogenic effect of therapeutic or diagnostic irradiation, as well as of the increased radiation background, has not been settled yet, but there is some evidence that increased exposure to x-ray increases the incidence of leukemia.

Studies[751, 752] on people exposed to atomic bomb radiation in Hiroshima and Nagasaki revealed 75 cases of leukemia, indicating a great increase in incidence of this disease in the survivors of this exposure. The predominant form was chronic myelocytic leukemia, but it has been pointed out that lymphocytic leukemia is rare in Japan. The leukemogenic effect of ionizing radiation was distributed equally in all age groups and in both sexes. In some of the cases there was a prolonged latent period of several years before development of the disease. In these preclinical stages, disturbances of the red cells and platelets as well as of the leukocytes were noted. An increase in basophils, many of which were abnormal, was one of the outstanding features. Lange, Moloney, and Yamawaki hypothecate that disturbances in cell enzyme systems with a resultant loss or alteration of cell growth-promoting or regulating factors account for the leukemic changes.

Table 271

Myeloblasts	*Promyeloblasts*	*Basophilic myelocytes*	*Basophilic segmented cells*	*Eosinophilic myelocytes*	*Eosinophilic juvenile cells*	*Eosinophilic stab cells*
10.2	6.2	0.6	1.4	2.2	0.4	0.6

Incidence: All statistical figures point to an increase in leukemias.[756] Part of this increase is accounted for by improvement in diagnosis and part of it is due to the increasing percentage of older people in the population who fall victim to chronic lymphatic leukemia. There still remains the increased frequency of acute leukemia in the ages between 2 and 5 yr.[757]

Red cells and hemoglobin in leukemia: Naegeli states that polycythemia is always present in mild cases of leukemia, but this is only for a very brief period. Anemia appears, with hemoglobin falling to 60% and red cells decreasing to 3,000,000. As the disease progresses, the anemia becomes more severe. There is an increase in reticulocytes, and many normoblasts appear in the picture. The anemia may be due to crowding out of the erythrocytic elements by the proliferating white cells, or it may be due to hemolysis or hemorrhage. Bone marrow depression, hypoplasia, or maturation arrest are seldom responsible for the anemia, but if they are present, they are due to hypersplenism. Granulocytic leukemia seldom gives rise to autoimmune hemolytic anemia which is frequently seen in chronic lymphatic leukemia. Hemorrhage is seldom an important feature of chronic leukemias.

In the terminal stage of chronic myelocytic leukemia the anemia becomes more marked, as a result of which the patient usually dies. The picture at this point is very much like that of a true pernicious anemia.

Types: There are 2 main types of leukemia—acute and chronic. This classification is based not only on the clinical course but also on the histologic cell type, the more immature forms being associated with the acute forms of leukemia. It is obvious that in some cases the clinical course will belie the histopathologic classification. It is customary to classify the leukemias further on the basis of the predominating cell type into 3 classes: (1) myelocytic (granulocytic), (2) lymphocytic, and (3) monocytic. Other forms of leukemia have also been described: plasma cell leukemia, basophilic leukemia, eosinophilic leukemia, megakaryocytic leukemia, hemocytoblastic leukemia, stem cell (blast cell) leukemia, and erythroleukemia. As will be shown later, these last diseases are subgroups of the main types of leukemia.

On the basis of the number of leukemic cells found in the peripheral blood, leukemia can be divided into leukemic, subleukemic, and aleukemic forms. In the leukemic form there is leukocytosis showing numerous abnormal cells. In the subleukemic form there is leukopenia but a number of leukemic cells can be seen, whereas in the aleukemic form there is leukopenia, and no leukemic cells can be found in the routine peripheral blood film. A buffy coat preparation would probably reveal their presence.

Chronic leukemias

Chronic myelocytic leukemia

Hematologic findings: Chronic myelocytic leukemia is characterized by highly active hyperplasia of the bone marrow which floods the blood stream with all the varieties of mature and immature leukocytes of marrow origin. In this disease there are a great many white cells, chiefly the immature forms of the granular leukocytes, together with a progressive anemia showing many varieties of nucleated red cells. The name leukemia was given by Virchow to this disease because of the appearance of the blood at autopsy, namely, whitish, but this appearance is never seen during life.

The total white count is usually about 100,000/c.mm., but it may go as high as 1,000,000. When the count is very high, it is necessary to use the red blood counting pipet in making the white count.

The differential count of leukocytes varies in different patients. The neutrophil is the predominant cell in some cases, but this is not always true. Eosinophilic leukocytes are constantly increased in actual numbers and percentage. Basophils may be increased up to 10-15% of a high total count. Myelocytes are always present; usually they are numerous, sometimes forming one-half of the total white cells in the blood picture. The greater the blood count, the greater are the number of myelocytes, especially in the later stages of

Eosinophilic segmented cells	*Neutrophilic myelocytes*	*Neutrophilic juvenile cells*	*Neutrophilic stab cells*	*Neutrophilic segmented cells*	*Lymphocytes*	*Monocytes*	*Other cells*
3.8	16	12	8.6	36	0.8	1.0	0.2

the disease. Most of the myelocytes are neutrophilic, but there are usually eosinophilic and basophilic myelocytes as well. About 3-5% of the cells in the differential count are myeloblasts, with a few promyelocytes. There are usually less than 5% of lymphocytes in the total count. There may be an occasional monocyte. The myelocytes are often atypical, varying in size and number of granules. Often the neutrophilic myelocytes retain the blue-staining cytoplasm which designates younger cells. There are many intermediate forms between the myelocyte and the stab cells in this disease. These intermediate forms are called juvenile cells, but many of them are atypical. With the oxydase stain, one can easily recognize just how many cells in the blood film are myeloid in origin.

The white cells in granulocytic leukemia have a very low alkaline phosphatase and glycogen content, as proved by histochemical stains. Some of the myeloblasts may contain Auer bodies.

Rarely chronic granulocytic leukemia occurs in childhood.

The white cell count in the terminal stage may approach a normal figure due to complete asphyxiation of the bone marrow function. In the terminal stage increasing immaturity in cell forms is seen. So many myeloblasts are seen that the picture suggests an acute myeloblastic leukemia.

It must be remembered that transitions from a chronic myelocytic blood picture to an acute myeloblastic one will occur and are connected with unfavorable changes in the course of the disease. A relapse or a terminal condition in chronic leukemia is usually attended with an acute blood picture.

Remissions are spontaneous. Apparent recoveries take place in leukemia. These remissions seem to date from the appearance of some intercurrent infection such as pneumonia, erysipelas, or purulent infection and sometimes tuberculosis. It seems likely that in the presence of a stimulus to leukocytosis the marrow reverts for a time to normal and orderly leukopoiesis, returning later, of course, to its leukemic tendencies. These remissions sometimes last several months, but one must not be deluded into the thought during this period that the patient has been cured. Total count may drop from several hundred thousand to a normal figure following an acute infection, but sooner or later the count insidiously begins to rise. Later on, with the end of the remission, the total count rises, and the differential count becomes as characteristic as it was before. The red cell count, however, does not ever become normal during a remission.

Blood platelets: Blood platelets are increased to high figures. Minot and Buckman[758] reported as high as 2,000,000 blood platelets/c.mm. The blood platelet count drops in the terminal stage. It must be assumed that these platelets are functionally inadequate (thrombocytopathy), as patients may bleed despite the high platelet count.

Making differential count of blood in myelocytic leukemia: To make a differential count of blood in myelocytic leukemia, construct a recording sheet as shown on p. 1357 and proceed to count 500 leukocytes. The method of counting blood of cases of myelocytic leukemia is given here as a suggestion to enable technicians to make these counts with the least possible difficulty. Use the 4-field meander method of Schilling (p. 1157), counting 125 cells in each of 4 fields, and record the count in the usual way, extending the hemogram as shown in Table 272.

In reporting the count, divide the total results by 5 to convert to % or the number of different types of cells in 100 leukocytes. Forms of reports vary in different institutions, but where no laboratory form is at hand, the following is suggested (Table 271).

For every 100 leukocytes counted there were 0.8 megaloblast, 1 with karyorrhexis; 1.6 macroblasts, 2 with karyorrhexis; 8 normoblasts, 3 with karyorrhexis. One erythroblast in mitosis was found on the slide, and there were marked polychromasia, slight anisocytosis, and enlarged blood platelets.

Nonhematologic laboratory findings:

Basal metabolic rate: Marked elevation without evidence of thyroid disease.

Urine: Increase in uric acid.

Plasma: Increase in uric acid level; high serum level of vitamin B_{12}.

Differential diagnosis: The diagnosis of chronic granulocytic leukemia may at times be rather difficult, especially in the early stages of the disease.

The differential diagnosis includes the following: (1) myelophthisic anemia with or without myeloid metaplasia of the spleen (myelofibrosis, myelosclerosis, carcinomatosis), (2) agnogenic myeloid metaplasia, and (3) severe leukemoid reactions.

Helpful differentiating laboratory findings:

1. Level of total white blood cell count. This is highest in granulocytic leukemia.

2. Shift to the left in the differential count. This is usually more marked in granulocytic leukemia.

3. Alkaline phosphatase stain. Alkaline phosphatase is normal or increased in leukemoid reactions and in myelophthisic anemia.

Table 272. *Suggested recording sheet for differential counting of blood films of myelocytic leukemia*

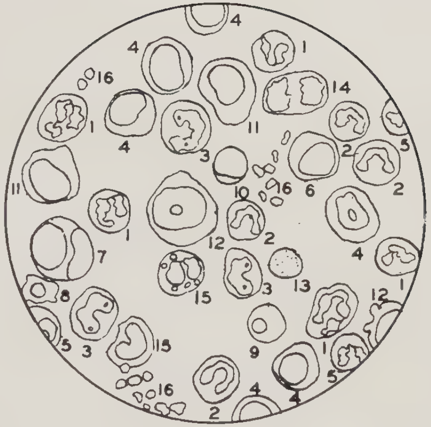
	25	*50*	*75*	*100*	*125*	*150*	*175*	*200*	*225*	*250*	*275*	*300*	*325*	*350*	*375*	*400*	*425*	*450*	*475*	*500*	TOTAL	%
Myeloblasts																						
Promyelocytes																						
Basophilic myelocytes																						
Basophils																						
Eosinophilic myelocytes																						
Eosinophilic juvenile cells																						
Eosinophilic stab cells																						
Segmented eosinophils																						
Neutrophilic myelocytes																						
Neutrophilic juvenile cells																						
Neutrophilic stab cells																						
Segmented neutrophils																						
Lymphocytes																						
Monocytes																						
Reticuloendothelial cells																						
Plasma cells																						
Other forms																						

Polychromatic megaloblasts . . . . . karyorrhexis . . . . . mitosis
Orthochromatic megaloblasts . . . . . karyorrhexis
Polychromatic macroblasts . . . . . karyorrhexis
Orthochromatic macroblasts . . . . . karyorrhexis
Polychromatic normoblasts . . . . . karyorrhexis
Orthochromatic normoblasts . . . . . karyorrhexis
Polychromatic microblasts . . . . . karyorrhexis
Orthochromatic microblasts . . . . . karyorrhexis

Anisocytosis________Poikilocytosis________Polychromasia________Basophilic punctation________Double nuclei without signs of mitosis________

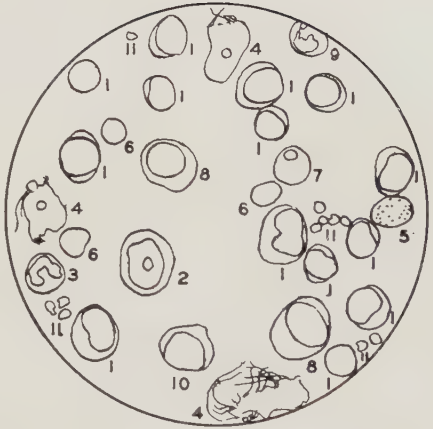
Other changes____

Other abnormalities____



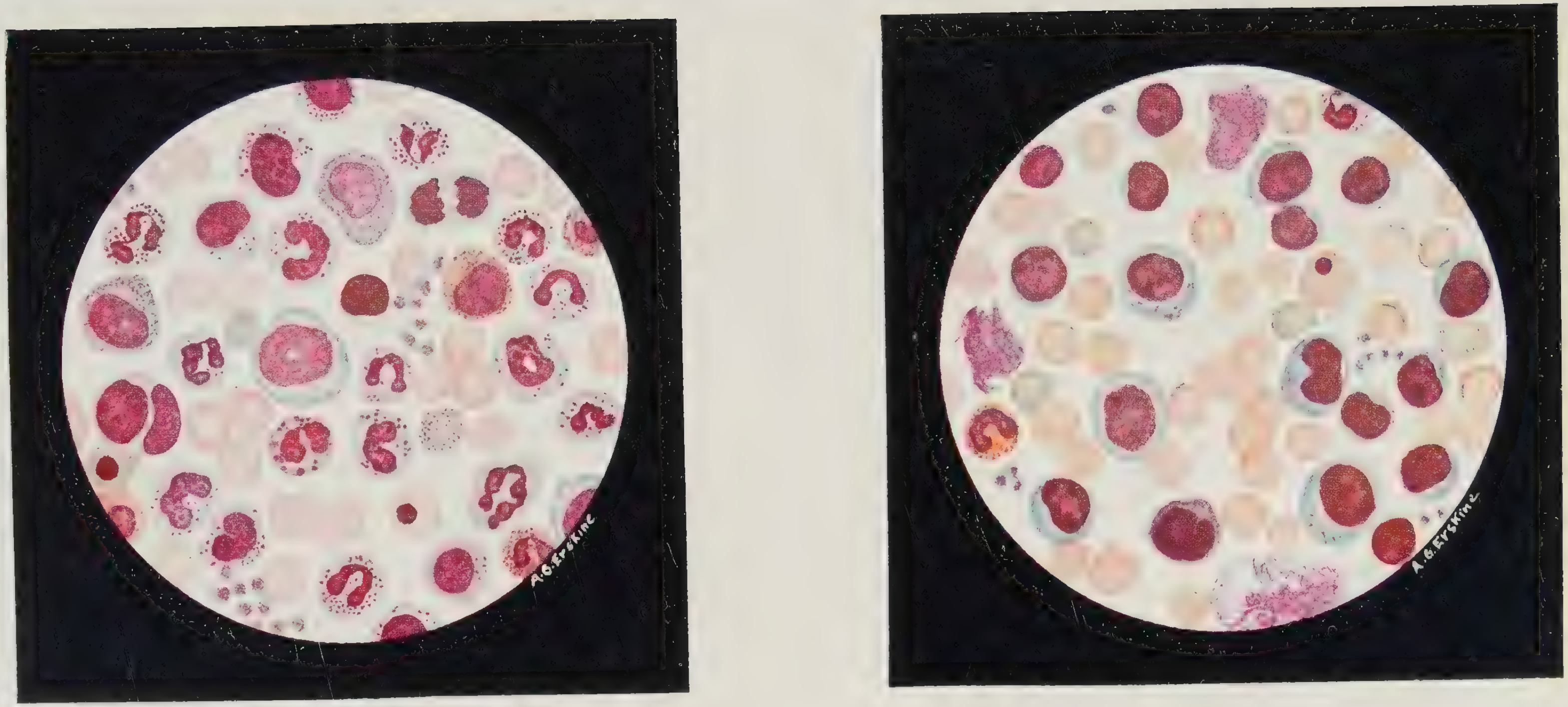
Chronic myelocytic leukemia
(Giemsa stain; ×950)

1. Segmented neutrophils
2. Stab cells
3. Juvenile cells
4. Neutrophilic myelocytes
5. Eosinophils
6. Eosinophilic myelocyte
7. Myeloblast with 2 nuclei without signs of mitosis
8. Polychromatic normoblast
9. Orthochromatic normoblast
10. Lymphocyte
11. Promyelocytes
12. Myeloblasts
13. Basophilic punctated erythrocyte
14. Erythroblast undergoing mitosis
15. Basophils
16. Blood platelets



Chronic lymphocytic leukemia
(Giemsa stain; ×950)

1. Lymphocytes
2. Immature lymphocyte
3. Eosinophil
4. Fragmentary nuclear structures
5. Basophilic stippled erythrocytes
6. Polychromatic erythrocytes
7. Normoblast
8. Large lymphocytes with azurophilic stippling
9. Segmented neutrophil
10. Monocyte
11. Blood platelets


A.G. Erskine
A.G. Erskine

Chronic myelocytic leukemia

Chronic lymphocytic leukemia

Fig. 443

Alkaline phosphatase is decreased or absent in leukemia.

4. Shape and size of red blood cells. In granulocytic leukemia and in leukemoid reactions the red cells are usually normocytic and normochromic.

5. Bone marrow aspiration or surgical biopsy. This is the best way to establish the diagnosis of myelophthisic anemia.

Chronic lymphocytic leukemia

Chronic lymphocytic leukemia is a disease of middle and later life rare under 20 yr. of age and most common after 50 yr. of age. The pathologic change is in the lymphatic tissues. The spleen is not usually as enlarged as in myelocytic leukemia. The spleen is engorged with lymphocytes and often shows areas of infarction. The bone marrow shows replacement of fat and of normal red cell and myeloid cell tissues by lymphocytic infiltration. The lymph nodes of the entire body are enlarged, including not only the superficial glands of the neck, axillae, groins, etc., but also the lymphoid follicles of the nasopharynx, tonsils, tongue, and gastrointestinal tract. The retroperitoneal and mediastinal nodes are often enlarged and cause symptoms by pressure. An anemia is present due to the crowding out of erythropoietic tissue by the lymphocytes. There is relative neutropenia.

The white cell count varies from 50,000-1,000,000, with an average of 100,000/c.mm. Eighty per cent or more of the cells in the differential count are lymphocytes of approximately the same size.* The remaining cells are neutrophils, including myelocytes, with a few eosinophils, basophils, or monocytes. Lymphoblasts and abnormal lymphocytic forms are seen in acute relapses. The red cell changes are those commonly seen in anemias associated with myelogenous or lymphatic dyscrasias. The blood platelets are reduced, and there is a slight prolongation of bleeding time.

Lymphocytic leukemia is likely to show a transition of the blood picture to the aleukemic state or even the acute state just as is seen in myelocytic leukemia. This occurs spontaneously, during the course of treatment, or because of the onset of an intercurrent infection.

Rather important is the benign asymptomatic form of chronic lymphatic leukemia in elderly patients. Only the peripheral blood may show leukemic changes, whereas the bone marrow remains normal.

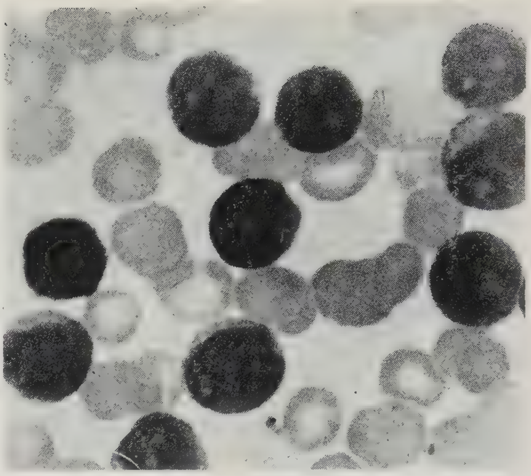


Fig. 444. Lymphocytic leukemia, blood film, showing crowding of lymphocytes. (×950.)

Nonhematologic findings: Abnormal proteins are frequently found in chronic lymphatic leukemia. The fibrinogen level and the titer of circulating fibrinolysins may be increased. The gamma globulin may show variation, extending over the entire spectrum of hypogammaglobulinemia to hypergammaglobulinemia. Autoimmune antierythrocytic and antileukocytic antibodies are frequently present, the first accounting for the hemolytic anemia commonly found in the disease. The occurrence of macroglobulins and cryoglobulins may make the differentiation from macroglobulinemia and from chronic plasmocytic leukemia somewhat difficult. The serum albumin and the antibody-producing fraction of the gamma globulins are usually decreased, leading to frequent infections and hemorrhages (even in the face of hypergammaglobulinemia). The basal metabolic rate suggests hypermetabolism.

Acute leukemias (myelocytic, lymphocytic, and monocytic)

Friedreich[759] reported a case of leukemia with an acute course. Ebstein[760] in 1889 described the clinical picture of acute leukemia. Fraenkel[761] in 1895 called attention to changes in the cells in the blood and claimed that all acute leukemias were of lymphatic origin because of the similarity of the cells to lymphocytes. Naegeli[762] in 1900 first described the myeloblast, furnishing the first data on which to classify 2 types of leukemia, myelocytic and lymphocytic. It remained for Schilling and Reschad[763] in 1913 to describe their classic

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*The monotonous blood picture.

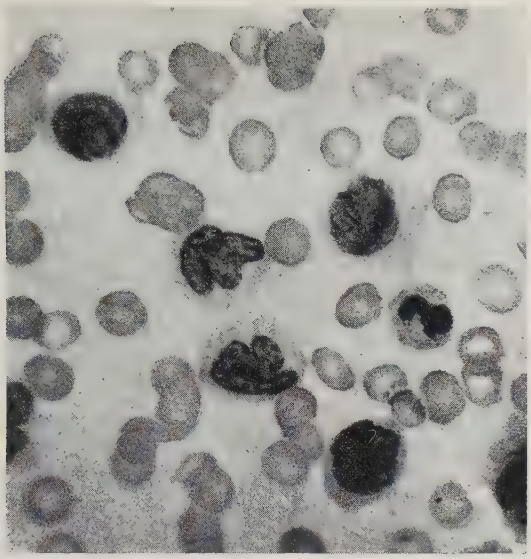

Fig. 445. Monocytosis, blood film, showing large number of monocytes. (×950.)

case of acute monocytic leukemia. This is considered the first case of this type reported or the first case of reticuloendotheliosis, as some prefer to call it. Up to the time of Clough's report in 1932[764] but 23 cases had been cited. In February, 1934, Forkner[765] reported that during the past 2 yr. 16 more cases of acute leukemia had been studied at the Thorndike Laboratory of the Boston City Hospital; these 16 cases were divided into the 3 main types—myelocytic, lymphocytic, and monocytic (6 of the last). Post-mortem examinations were made in 4 of the 6 cases of monocytic leukemia; 1 case of myelocytic and 1 case of acute lymphocytic leukemia were included with autopsy findings.

Forkner called attention to certain clinical differences between acute monocytic and other forms of acute leukemia. He insisted that the clinical picture of diffuse, marked swelling of the mucous membranes, particularly of the gingivae usually with ulceration and necrosis, is characteristic of acute monocytic leukemia and is usually absent in acute leukemia of the other types. There was diffuse cellulitis around the lesions of the mucosa, causing swelling and pain with signs of acute inflammation extending into the deeper tissues of the face. It was for this reason **that all these patients with monocytic leukemia reported by Forkner consulted a dentist early in the course of their illness.** Although acute lymphocytic and myelocytic leukemia often have mouth lesions, they are different, consisting of hemorrhages and slight infection, not necrosis. This fact stands out prominently in the 23 cases reported by Clough[764]: 18 had marked stomatitis or pharyngitis and abscesses of the alveoli, and 4 had swelling of the neck. Forkner studied the case histories of 8 additional cases which were not accepted by Clough. Three cases reported by Ewald, Frehse and Hennig[766] in 1922, 1 additional case by Bingel[767] in 1916, 1 case by Dias[768] in 1930, 1 case by Hittmair[769] in 1930, and 2 cases by Asselstine[770] in 1932 showed lesions of the mouth similar to those seen in the other 18 cases tabulated by Clough.

There are other physical signs which serve to differentiate these cases. The spleen is somewhat enlarged but is not palpable in acute myelocytic leukemia, but in the acute lymphocytic type it is palpable 2-3 cm. below the costal margin. The spleen in acute monocytic leukemia according to Clough was palpable in 16 of the 23 cases recorded by him. In Forkner's 6 cases the spleen was palpable from 1-4 cm. below the costal margin. The weight of the spleens in the 4 cases studied by him at autopsy were 1065, 606, 360, and 900 gm., respectively.

The size of the lymph nodes should be used as an aid in the diagnosis of the type of acute leukemia. Forkner claims that there is invariably an enlargement of the lymph nodes in acute lymphocytic leukemia, but it is not as marked as in chronic lymphocytic leukemia. The lymph nodes, particularly those in the neck, may be slightly or moderately enlarged in acute monocytic leukemia, but we never see the enlargement in this disease that occurs in the acute lymphocytic type. There is only a negligible enlargement in acute myelocytic leukemia.

Acute leukemia in children is often accompanied by joint pains.

The clinical and histologic differentiation of the 3 types may be seen by studying Table 273, arranged by Forkner.

The characteristics above set forth will help the differential diagnosis, in the absence of a clear-cut blood picture or histologic study of the tissues at autopsy.

Blood picture: There has existed considerable confusion as to the cytology found in these 3 types of acute leukemia. This is enhanced by the fact that as many as 85% of the leukocytes in any case of acute leukemia are of a uniformly immature type, usually in the myeloblast, lymphoblast, or monoblast stage of development. Intermediate stages are few. There are, too, some immature types

Table 273. *Clinical and histologic differentiation of three main types of acute leukemia*

	Acute myelocytic leukemia	*Acute lymphocytic leukemia*	*Acute monocytic leukemia*
Spleen	Usually not palpable	Almost invariably significantly enlarged	Palpable in about 70% of cases
Lymph nodes	Usually slight or no enlargement	General enlargement of moderate or marked degree	Moderate in neck, but other lymph nodes very slightly enlarged, if at all
Liver	Usually palpable	Usually palpable 1-4 cm. below costal margin	Usually palpable 1-4 cm. below costal margin
Mucous membranes, particularly of mouth and pharynx	Petechiae, bleeding; often slight swelling of gingivae but rarely ulceration	Petechiae, bleeding; rarely ulceration	Petechiae, bleeding; usually marked, diffuse swelling of gingivae or pharynx with ulceration; often cellulitis with swelling and tenderness of face; marked fetor oris
Oxydase reaction	From few to many oxydase-positive cells, with from few to many coarse granules	All lymphoid cells oxydase-negative	
Histologic distinctions of type cells in blood and tissues Other characteristics	Dominant-cells myelocytes A (Sabin) and myeloblasts; nuclei round or oval, usually with several nucleoli; cytoplasm deeply basophilic; myelocytes B (Sabin) present; Auer bodies frequently present in few cells	Dominant cells lymphoblasts and young lymphocytes; nuclei round, oval, or slightly indented, with few nucleoli usually present; relatively small amount of deeply basophilic hyaline cytoplasm; mature lymphocytes present in fair number; occasional myelocytes C (Sabin) and metamyelocytes present	Dominant cells monoblasts and premonocytes; nuclei usually elongated and folded, deeply indented, or otherwise irregular in contour; nucleoli rarely present; cytoplasm relatively abundant, usually not deeply basophilic and not hyaline but slightly basophilic and ground-glasslike; Auer bodies may be present in few cells; mature monocytes present in significant and varying numbers; myelocytes C and metamyelocytes often present in small numbers

present other than those of the typical leukemia with which we are dealing. Sabin,[771] Simpson,[772] and others, however, have rediscovered a method of aiding in the recognition of these cells, namely, the supravital method. One must not forget, too, that the oxydase test, as used by Schilling and his followers, is of paramount importance in this regard. Forkner, with illustrated charts of cells found in patients, called attention to some striking points of identification in regard to myeloblasts. The nuclei are round, indented, or oval and contain several nucleoli. The cytoplasm is basophilic and of uniform character. The cells in monocytic leukemic cases are more mature and possess nuclei of complicated characteristics, with very few nucleoli; their cytoplasm is relatively abundant, is basophilic, and contains dustlike granules.

Histologic differentiation of acute leukemias: The oxydase test for blood cells is a method for the differentiation of the tissues at autopsy in all leukemias. This is a helpful criterion for the pathologist. Cells infiltrating the liver, kidney, etc., if oxydase-positive point to the myelocytic character of the case. If they are oxydase-negative, it is a case of lymphocytic leukemia or lymphoblastoma. In monocytic leukemia the oxydase reaction may be positive or negative. Forkner calls attention to certain definite means of distinguishing monocytic cells in paraffin sections. Under the oil immersion objective, they show characteristics that will distinguish them from myeloblasts or lymphoblasts. The nuclei even of promonocytes and of some monoblasts are irregular in shape, in the form of an irregular crescent or a broad elongated nucleus bent upon itself. The nucleus is bent on itself several times, giving the impression of lobulation. This point made by Forkner is very impressive, especially since he has so insistently called attention to the "lobulation" of the nuclei of monocytes. May it not be true that this is not a lobulation but a pseudolobulation, as pointed out by Forkner? Distinct separation of the lobules is not demonstrable, in contrast to the ease with which it is seen in the segmented neutrophil. There are also many young monocytic cells, with simple round, oval, or slightly indented nuclei, but these cells exist as close companions of the cells with the more complicated nuclei as described. Furthermore, there are other characteristics which serve to distinguish the monocytic cells from the early lymphoid and myeloid elements. The nuclei of the monocytic series of cells rarely have demonstrable nucleoli, whereas these structures are common in lymphoblasts and myeloblasts. The cytoplasm of promonocytes and monoblasts is as a rule more abundant and much less basophilic than that of either lymphoblasts or myeloblasts and frequently it is so faint, with hematoxylin, that the border of the cell is barely recognizable. This is in contrast to the moderate or very deep basophilia of early lymphoid and myeloid cells.

In reference to the terms acute and chronic lymphocytic leukemia, Wiseman[773] has introduced the thought that from a hematologic point of view responses of the lymphatic tissue vary to such a degree that the terms acute and chronic are inadequate to express this change. Neither do these terms take into account the important relationship of lymphosarcoma to leukemia. "Finally," he states, "there does not appear to be any justification for the term 'aleukemic lymphatic leukemia,' since there is no fundamental pathologic difference between cases presenting low and high total white blood cell counts."* He showed records that indicated that lymphocytic leukemia may be a very benign disorder which does not materially shorten life or measurably diminish health. On the other hand, it may kill quickly. It is probable that there are many more cases in the former group than one would suspect since many are asymptomatic, do not show physical signs, and hence are not seen by the physician.

Monocytic leukemia (leukemic reticuloendotheliosis)

Monocytic leukemia is a rather rapidly fatal disease showing increased numbers of monocytes in the blood stream and in other organs. It was first reported by Reschad and Schilling[774] in 1913. Rosenthal[775] reported the first case in America in 1921. A large number of cases has since been reported, 127 in all in the American literature up to June, 1937, and many cases of this disease are on file in the Registry of Blood Diseases of the American Society of Clinical Pathologists. Osgood[776] reported 6 cases and Rosenthal and Harris and Doan and Wiseman found reports of 532 cases of leukemia, from which statistics on the incidence of monocytic leukemia were calculated. It was estimated that 62.2% of all cases of leukemia reported in the literature were cases of myelocytic leukemia, 32.7% were cases of lymphocytic leukemia, and 5.1% were cases of monocytic leukemia.

In considering this disease, the two schools of thought relating to the origin of blood cells must be taken into account. One school, of which Naegeli is a proponent, denies the reticuloendothelial source of the monocytic cells, and the other, of which Schilling is the leading proponent, insists that these cells originate from reticuloendothelial structures. It can no longer be denied that there are certain cases of monocytic leukemia which begin to all intents and purposes as myelocytic leukemia. These cases inevitably become cases of pure monocytic leukemia. Naegeli has claimed that monocytic leukemia is a temporary monocytic phase of myelocytic leukemia, and that the monocyte is a descendant of the myeloblast. He denied that monocytic leukemia is a distinct pathologic entity and cited cases which at one time exhibited the hematologic features of monocytic leukemia and in which the typical blood picture of myelocytic leukemia developed later. From such cases he claimed to trace intermediate stages between myeloblasts and monocytes. Merklen and Wolf,[777] Gittins and Hawksley, Reich, and others have reported cases supporting Naegeli's views. On the other hand, Schilling and his followers have maintained that monocytic leukemia is a distinct pathologic entity in which there is monocytosis in the circulating blood and hyperplasia of the reticular tissue in the hemopoietic organs. Schilling maintains that this disease is separate from myelocytic and lymphocytic leukemia. Forkner[765, 778, 779] believes that monocytes and their precursors are not a part of the reticuloendothelial system and that there is no clear-cut evidence of any intimate relationship between monocytes and the reticuloendothelial system, if the latter is considered in its strict sense. He likewise claims that any relationship of monocytes to myeloblasts has never been

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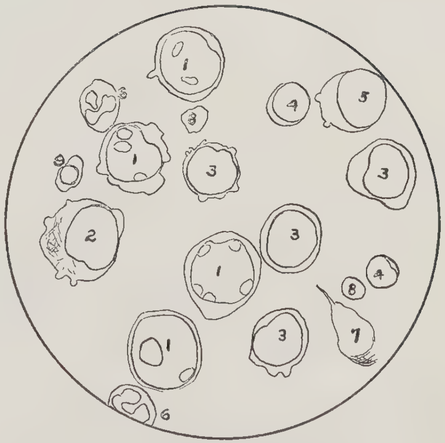
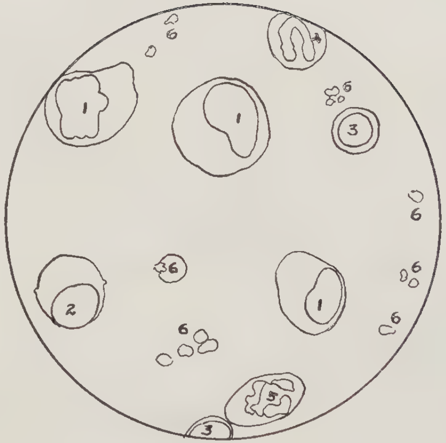
*From Wiseman, B. K.: J.A.M.A. **107**:2016, 1936.



Acute leukemia
(Blood film; Giemsa stain; ×950)

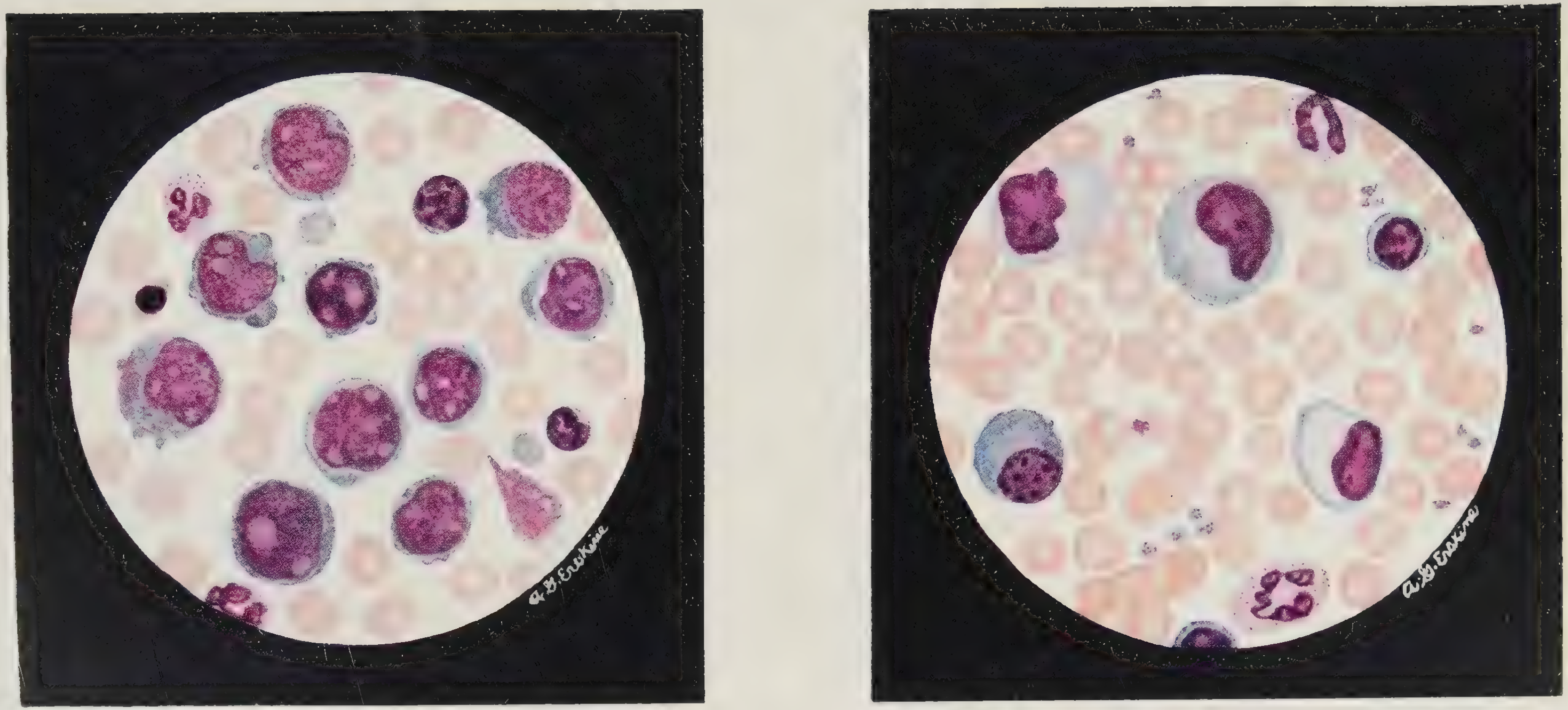
1. Lymphoblasts
2. Prolymphocyte with Auer rods
3. Atypical young lymphocytes
4. Small lymphocytes
5. Plasma cell
6. Segmented neutrophil
7. Fragmentary nuclear structure
8. Polychromatic erythrocyte
9. Normoblast

Note lack of blood platelets



Infectious mononucleosis
(Blood film; Giemsa stain; ×950)

1. Atypical large lymphocyte
2. Plasma cell
3. Small lymphocyte
4. Neutrophilic stab cell
5. Segmented neutrophil
6. Blood platelets


A. G. Erskine
A. G. Erskine

Acute leukemia

Infectious mononucleosis

Fig. 446

proved, and he does not believe that the type of leukemia ever changed, for example, from monocytic to myelocytic leukemia.

It is for the reason that undoubted cases of myelocytic leukemia changing to monocytic leukemia have been reported by reliable workers that it is necessary to divide monocytic leukemic cases into 2 types: first, the monocytic leukemia of the Schilling order and, second, the monocytic leukemia of the Naegeli type.[780]

Monocytic leukemia of Schilling type

Monocytic leukemia may occur at any age, the youngest patient reported being 11 mo. old and the oldest 78 yr. of age. The clinical course runs about the same as the other acute leukemias, lasting from 2 weeks to a few months. Infection of the mouth or throat and spontaneous purpura are commonly seen. Swelling of the gums is a constant feature and is more marked and constant than in other cases of acute leukemia. This swelling is associated with gangrenous stomatitis. There is enlargement of the spleen and lymph nodes. Enlargement of the liver is also common, having been observed in 66% of the 90 cases in which a notation was made. Other clinical symptoms are pallor, weakness, and fever. There are also cutaneous lesions of 2 types: firm and painless nodules in the skin, which on histologic examination prove to consist largely of monocytes, promonocytes, monoblasts, and reticular tissue, and staphylococcic infection of the skin, with multiple boils and carbuncles.

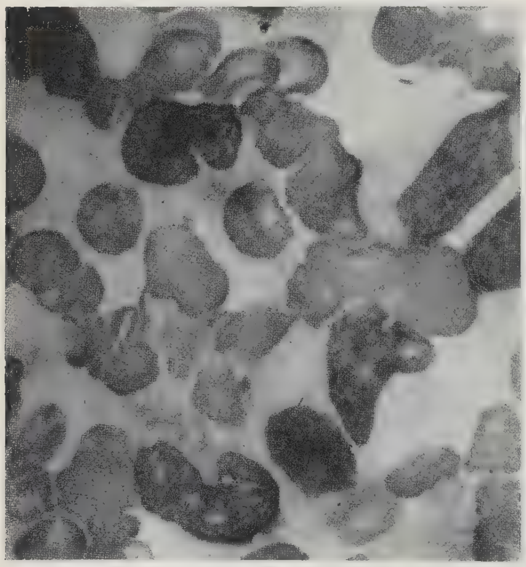


Fig. 447. Endocarditis, blood film, showing endothelioid monocytes. (×950.)

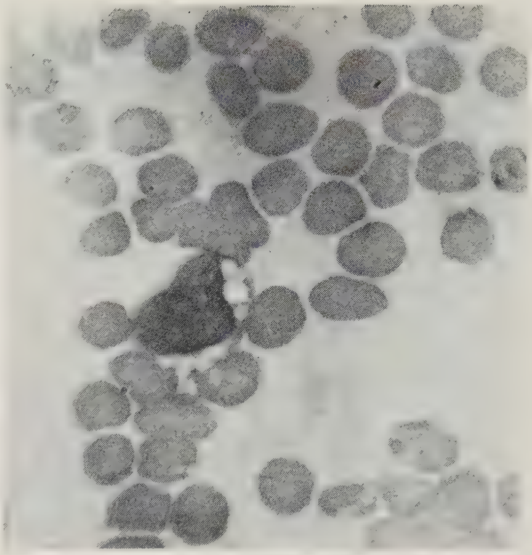


Fig. 448. Blood film showing a phagocyte. (×950.)

Blood picture: The blood picture shows a marked or moderate decrease in red cells and hemoglobin. There is a definite increase in the white cell count. The average of the highest counts recorded in each case was 99,600. A few cases showed leukopenia. Differential count showed mature monocytic cells forming about 50-75% of the total count. Sometimes immediate precursors of the monocyte, including the histiocytes, are present. The cytoplasm resembles that of the mature monocyte, being of blue-gray color and of ground-glass appearance, with typical fine monocytic granules which are first seen about the nucleus in the youngest cells. The nucleus is large, oval, bean-shaped, or even lobulated. The chromatin is arranged in a very fine spongelike meshwork which becomes coarser and skeinlike in more mature cells. Although most of the observers report that the immature cells are oxydase-negative, Farley[781] and Lawrence[782] and co-workers found the cells to be oxydase-positive.

Histologically, the architecture of the lymph nodes and spleen is obliterated by a marked proliferation of reticular cells. Periportal growth is seen in the liver, and proliferation of reticular cells is found in the kidneys, the serosal surfaces, and the perivascular stroma of nearly all the organs, especially the adrenal glands, thymus, pancreas, and gastrointestinal tract.

The **bone marrow** shows a preponderance of monocytic cells. These are monoblasts which are very large, from 15-30μ in diameter. Chromatin structure of the nucleus is very fine, similar to that of the myeloblast but even more transparent. The nucleus is usually round or oval. The cytoplasm ranges from basophilic to deeply basophilic, and there is more in proportion to the size of the nucleus than in the myeloblast or the lymphoblast.

As Osgood pointed out, monoblasts are difficult, sometimes impossible, to distinguish from myeloblasts or lymphoblasts. The chief difference is the more frequent occurrence of fine, diffuse azurophil granules in the cytoplasm of the monoblast, similar to those of the mature monocyte. Peroxidase stain does not assist in this differentiation, as all these cells may show a negative peroxidase reaction. From his study of 6 cases and a review of the literature, Osgood definitely believes that the monocyte is developed from a cell which may be called the monoblast, similar in morphology to the myeloblast. This is very interesting in view of the fact that Forkner has listed 19 different views as to the origin of the monocyte, which clearly shows the confusion that exists in regard to the origin of this cell. It is gratifying to know that a hematologist of Osgood's experience has so definitely accepted the entity of the monocyte as so determinedly maintained by Schilling and his followers all these years.

Monoblasts frequently contain Auer bodies.

Monocytic leukemia of Naegeli type

A report by Hall and Watkins[783] may be taken as an example of the monocytic leukemia of the Naegeli type, i.e., beginning as myelocytic leukemia and changing to monocytic leukemia. Hall and Watkins believe that a "monocytic phase" may develop at any time in the course of myelocytic leukemia or that the picture may be that of monocytic leukemia when the patient is first seen, later changing to one of chronic myelocytic leukemia which at a later date changes to one of monocytic leukemia, as was demonstrated by the case reported. Levine[784] reported a case which was studied for a long time because of severe anemia of indeterminate type. Ten days before death, a leukemic blood picture developed in which myeloid immaturity toward the stem cell and mature and young monocytes were observed, with the findings at necropsy of subacute myelocytic leukemia. Levine expressed the opinion that this picture illustrated the development of a monocytic phase in the course of myelocytic leukemia after an initial aleukemic phase.

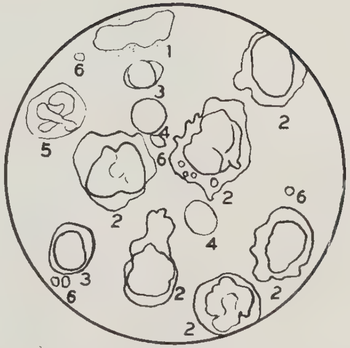
Hall and Watkins studied all of the cases of monocytic leukemia seen at the Mayo Clinic in a 10-year period and found that 23 cases were of the Naegeli type and 6 were of the Schilling type. They believe that the Naegeli type of monocytic leukemia should be considered a variant of myelocytic leukemia and the Schilling type a variant of leukemic reticuloendotheliosis.

There have been a number of erroneous conclusions about monocytic leukemia due to the fact that only single observations have been made on some cases. Thus at various times the features of agranulocytosis, aleukemic myeloid leukemia, aplastic anemia, leukemoid monocytosis, leukopenic infectious monocytosis, chronic splenic panhematopenia, and reticuloendotheliosis have been depicted. Monocytic leukemia may pursue a very erratic course. This was exemplified in the unusual case reported by Rappoport and Kugel.[785] This case was exhaustively studied. There were 4 sternal punctures. There were several episodes of granulocytopenia. Serial blood counts were made, and autopsy findings were very complete in this case. The conclusion was drawn that this was unquestionably a case of true monocytic leukemia. The authors believe that the distinction between the Schilling and Naegeli types of monocytic leukemia is more apparent than real and does not deserve the rigid segregation frequently employed and heretofore alluded to in this chapter. A similar contention has been advanced by Campbell et al.[786] and Custer.[787] This case illustrated, as do most of the cases, that in the later stages of the disease as the frank monocytic leukemia progresses the myeloid reaction diminishes. This myeloid reaction is for the most part an expression of the bone marrow reactivity to the agranulocytosis and is not an expression of the monocytic leukemia.

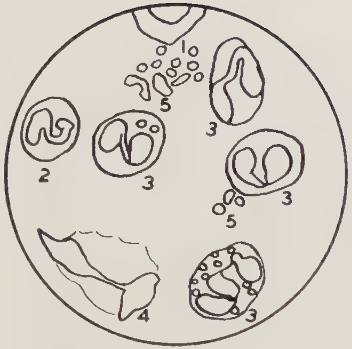
There are certain essential facts which must be borne in mind relative to the diagnosis of this disease. One of these facts is the proper identification of the monocyte. It is usually larger than the granulocyte; its cytoplasm is opaque and mottled, has a gray-blue color, and contains azurophilic granules. The cell margins are serrated and irregular. The nucleus is reniform or horseshoe-shaped and possesses a lacy, skeinlike, coarse chromatin weave. With supravital stain numerous neutral red or salmon-pink colored bodies, vacuoles, are symmetrically arranged around a centrosphere located in the bay of the nucleus. This structure is the rosette which is considered so typ-

Monocytic leukemia

1. Endothelioid monocyte
2. Monocytes
3. Lymphocytes
4. Polychromatic erythrocytes
5. Segmented neutrophil
6. Blood platelets



Monocytic leukemia
(Blood film; Giemsa stain; ×950)



Trichinosis
(Blood film; Giemsa stain; ×950)

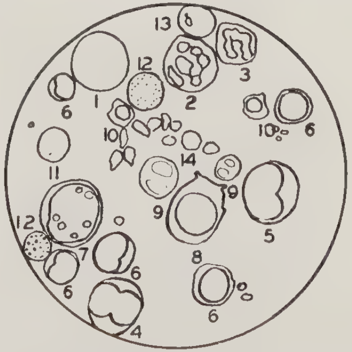
Trichinosis

1. Monocyte
2. Neutrophilic stab cell
3. Eosinophils
4. Crushed eosinophil
5. Blood platelets

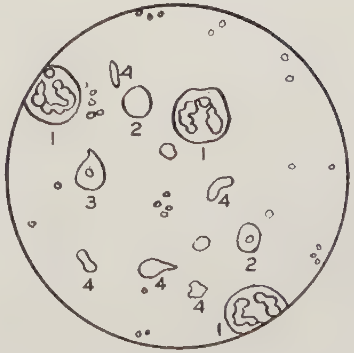
von Jaksch's disease

1. Megalocyte
2. Segmented neutrophil
3. Neutrophilic stab cell
4. Neutrophilic juvenile cell
5. Neutrophilic myelocyte
6. Lymphocytes
7. Lymphoblast or myeloblast
8. Plasma cell
9. Polychromatic erythrocyte with endoglobular degeneration
10. Polychromatic microblasts
11. Polychromatic erythrocyte
12. Erythrocytes with basophilic punctation
13. Blood platelet on a red cell
14. Blood platelets

Marked anisocytosis



von Jaksch's disease
(Blood film; Giemsa stain; ×950)

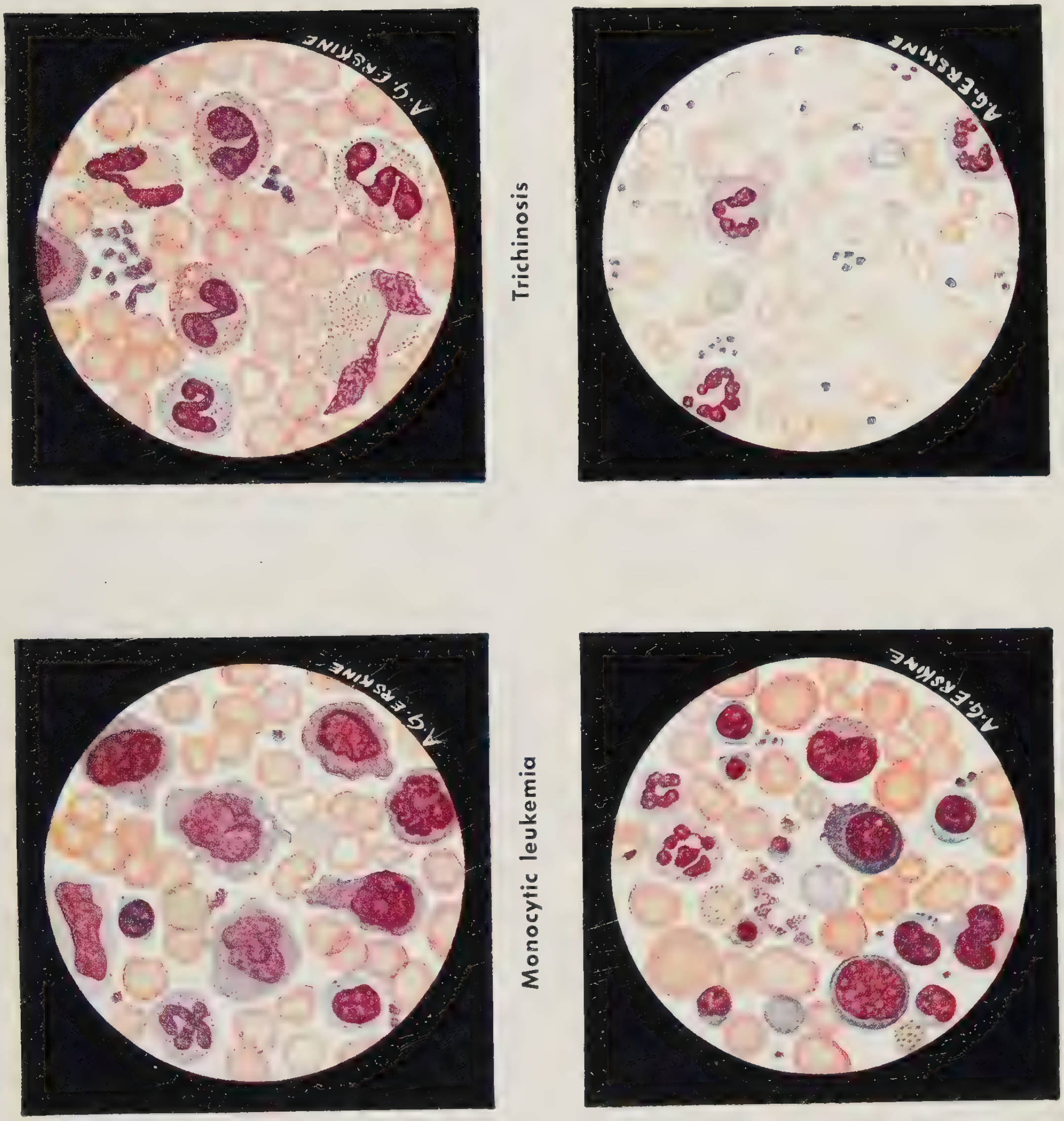


Chlorosis
(Blood film; Giemsa stain; ×950)

Chlorosis

1. Segmented neutrophils
2. Polychromatic erythrocytes
3. Poikilocyte with endoglobular degeneration
4. Poikilocytes

Hypochromic microcytic anemia
Marked poikilocytosis and anisocytosis



Trichinosis

Chlorosis

Monocytic leukemia

von Jaksch's disease

Fig. 449

ical of the mature monocyte. Moreover the monoblast measures from 15-20μ in diameter, and in fixed stained films it possesses a basophilic cytoplasm containing no granules and, distinctively, no Auer bodies. The nucleus is round, large, and centrally situated; its chromatin is fine, stippled, and sievelike and usually possesses two nucleoli.

Rappoport and Kugel emphasize that this diagnosis requires prolonged and intensive clinical and laboratory observations.

Lymphocytic leukemia following benzol poisoning

Falconer[788] reported a case of chronic benzol poisoning, gradually developing the picture of a typical leukemia as follows.

> The patient, a man 58 yr. of age, was employed in a can factory. The can ends, rimmed with a solution of rubber in benzol, were delivered to him by a conveyor, the benzol evaporating from the rubber mixture en route. Falconer had seen 4 cases of typical benzol poisoning from the same plant during the previous year. This patient's blood studies showed 50% hemoglobin, 1,530,000 red blood cells, 8100 white blood cells, 62% neutrophils, 32% lymphocytes and 6% monocytes. Blood platelets numbered 68,000; red cells showed anisocytosis and some polychromasia. The symptoms were diagnosed as chronic benzol poisoning. Later on hemorrhages from the nose began, together with decrease in red and white counts. After a blood transfusion, the nasal bleeding ceased. About a month later the patient returned for treatment, and 2 mo. later the bleeding from the nose reappeared. The blood picture showed decrease in the number of red cells and amount of hemoglobin, but the white cell volume rose. About 4 mo. later, he showed a picture of beginning lymphocytic leukemia, the counts running up to 50,000. This state persisted for about a year when death occurred, with a clinical diagnosis of lymphocytic leukemia.

Three important questions were suggested by a study of the clinical data and autopsy material on this patient. The autopsy material, by the way, showed general lymphadenopathy, increase in size of the spleen, leukemic infiltration of the liver and kidneys, secondary anemia, bronchopneumonia, and purulent bronchitis. The lymph nodes showed a striking overgrowth of tissue by a type of cell which was larger than the usual lymphocyte and which contained a thin rim of cytoplasm. The bone marrow showed the same type of cell as the lymph node.

The questions are as follows:

1. What effect does benzol have on lymphatic tissue?
2. Is it not possible for benzol, like other well-known hemopoietic irritants, to upset the hemopoietic balance between cell growth and destruction, giving rise to lymphoid hyperplasia?
3. Does this lymphoid hyperplasia always stop just short of leukemia, or can it under prolonged irritation or in susceptible individuals progress to lymphocytic leukemia?

Lignac[789] injected mice with from 25-30 injections of benzol (0.1 ml. of a solution of 0.3 ml. benzol in 10 ml. olive oil) and concluded that the symptoms of hyperplasia developed and that the spleen, which was first hypertrophied, decreased in size. The animals showed hyperplasia but no lymphocytic leukemia. He did not succeed in producing lymphocytic leukemia. The hyperplasia he considered a tumorous growth similar to leukemia. It is a real tumor of precursors of white blood cells. He stated that the immaturity of undifferentiated cells suggests acute leukemia, and the development of a tumor may or may not be accompanied by a leukemic blood picture.

Murphy and Sturm[790] reported experimental work with hemopoietic irritants accomplished by subjecting mice, rats, and guinea pigs to dry heat at temperatures of from 55°-65° C. with short exposures of about 5 min. White cell counts were made at intervals over a 3 wk. period. The blood picture showed an immediate drop in both neutrophils and lymphocytes, which averaged about 3000 cells/c.mm. Seven days after the heating there was an increase of from 5000-10,000 cells/c.mm., the increase being almost entirely in the lymphocytes. Fourteen days after heating, the lymphocytes had increased to from 12,000-14,000/c.mm. above the original counts; the neutrophil count remained low and did not increase until about 3 wk. after heating. From 6-10 days after heating several lymphocytes were noted apparently in the process of amitotic division. These cells in the process of division looked very much like the cells reported by Falconer in his case of leukemia following benzol poisoning.

Nakahara,[791] in trying to determine the source of lymphocytosis induced by heat, found the first change in the spleen and the lymph glands with widespread degeneration of the lymphoid elements. This was followed in 48 hr. by activity of the germinal centers, with an overproduction of lymphocytes, the spleen and lymph glands becoming larger than normal. There was a marked increase of mitotic figures in the lymphocytes of the germinal centers following the general necrosis.

Benzol presumably may act on lymphatic tissue in a manner similar to dry heat, causing destruction first then a compensatory hyperplasia exceeding the normal limits. It is questionable whether this might not later result in leukemia.

Experimental work carried on at the Rockefeller Institute[792, 793] shows that with a certain dose of roentgen rays lymphocytosis similar to that produced by heat can be brought about, i.e., first a lymphocyte fall, followed by a marked rise.

Basophilic leukemia

There is some dispute as to whether or not there is actually an entity which may properly be called basophilic leukemia. Many contend that these are cases of chronic myelocytic leukemia with large numbers of basophils. Groat and others[794] reported a case of acute basophilic leukemia which seems to bear out their belief that this entity may actually occur. They cite the report of Joachim[795] who in 1906 reported 2 cases of chronic leukemia in both of which the percentage of mast cells or basophilic cells was above 50. The blood picture in Joachim's case was of an ordinary chronic myelocytic leukemia except for the large number of mast cells, as high as 83%. Joachim's opinion was that the general symptoms were similar to other chronic myelocytic leukemias except for the degree of basophil increase. He reported it as a case of atypical leukemia, concluding that chronic basophilic leukemia is a variety of the common myelocytic variety. Sabrazès[796] in reporting a case of chronic myelocytic leukemia with many mast cells stated that the so-called hemohistioblast of Ferrata and many types of basophils were frequently found increased in chronic myelocytic leukemia. Elliott and Young[797] reported a case of chronic mast cell leukemia because of a predominance of leukocytes with coarse basophilic granulation.

Casey et al.[798] discussed what has already been stated in this book, namely, whether there is a true basophilic leukemia distinguishable from the neutrophilic type. Doan and Reinhart[799] reported 3 cases which they believed to be true basophilic leukemia. They seemed to believe that these were the first instances of this sort to be reported.

The case reported by Casey et al., which records the sixth case in the literature with clinical observations for at least 1 month before death, emphasized that there is no evidence at present that basophilic leukemia ever occurs except as a terminal phase of neutrophilic leukemia. They made a very proper recommendation for future observers, namely, that only those cases should be reported as basophilic leukemia in which (1) blood studies using supravital and peroxidase stains, together with routine blood examinations, have been made, (2) hematologic observations on the patient have been available for more than 3 months prior to death, and (3) postmortem examination with supravital studies of spreads from the bone marrow, blood, spleen, lymph nodes and alcohol fixation of the tissues for the preservation of the water-soluble basophilic granules have been made.

The relationship of blood basophils to the tissue mast cells and their functions has been considered in the discussion of basophils and mast cells. Cases of mast cell leukemia have been reported as well as several cases of urticaria pigmentosa associated with systemic involvement (mastocytosis).

Eosinophilic leukemia

Eosinophilic leukemia represents a rare form of chronic myelogenous leukemia and must be carefully separated from the more common eosinophilic leukemoid reaction. In order to establish the diagnosis of eosinophilic leukemia the peripheral smear must contain such immature cells as eosinophilic myelocytes and promyelocytes and myeloblasts. The finding of Auer bodies, Rieder cells, and anisocytosis (giant forms) of the eosinophilic granulocytes is very helpful. Leukemic eosinophils frequently exhibit irregular granulation since all the granules may not stain with acid dyes but accept basic dyes, thus masquerading as basophilic granules.

Plasma cell leukemia

In the literature there have been 62 cases of plasma cell leukemia reported. Foa[800] reported the first case. He called it pseudoleukemia plasmacellularis. He found plasma cells in the blood and diffuse infiltration of the bone marrow, liver, spleen, and some lymph nodes. Moss and Ackerman[801] reported a case in September, 1946. This man showed symptoms of swelling of the lower jaw, bleeding gums, and enlargement of the spleen and liver. The blood picture showed 13,700 leukocytes, with a differential count of 4% myelocytes, 14% juvenile cells, 15% stab cells, 24% segmented neutrophils, 23% lymphocytes, 6% monocytes, 2% eosinophils, and 1% basophils, with 11% plasmacytes, 4 pronormoblasts, and 24 normoblasts. The sternal bone marrow showed the marrow completely replaced by plasma cells. Diagnosis of plasma cell leukemia was made. Plasma cells reached a maximum value of 22%. There was a hyperglobulinemia throughout the disease. Autopsy 1½ months after admission to the hospital showed splenic enlargement, liver enlargement, and microscopically plasma cells present in every region of the liver. Spleen sinuses were plugged with plasma cells. Lymph nodes showed invasion of plasma cells, likewise the pancreas. Bone marrow showed a complete replacement of normal elements by a diffuse infiltration of plasma cells.[1]

Plasma cell leukemia represents the leukemic phase of multiple myeloma, a disease which can be considered to be a subleukemic or aleukemic leukemia. Its leukemic phase is rather uncommon.

In reference to clinical findings in this disease, it is usually found between the ages of 45 and 60 yr. The symptoms of plasma cell leukemia in the majority of cases are those of multiple myeloma. Pain in the arm, back, legs, or substernal region may be present, with a history of lassitude, weakness, and weight loss. There is a tendency to hemorrhage. Bleeding is nearly always from the gums and nose. The bone destructive nature of plasma cell leukemia is exemplified by frequent pathologic fractures. In the bone marrow there is diffuse infiltration with plasma cells. These cells do not differ from those in the blood, except that in the blood the plasma cells tend to be smaller. The liver is invaded by plasma cells and likewise the spleen and lymph nodes.

It should be stressed that, although we generally call these cells plasma cells, the term myeloma cells is distinctly preferable. In multiple myeloma and plasmacytic leukemia the proliferating cells are rarely mature plasma cells but usually differ from the mature forms by their large size, their variability in size, their variably stained cytoplasm (from deep blue to light blue and pink), their larger nuclei, the absence of perinuclear clear zones, their multinucleation, and the frequent presence of prominent nucleoli. The plasma cells found in the blood are usually much more mature than the ones infiltrating the bone marrow.

Askanazy[802] formulated the following requirements for a diagnosis of plasma cell leukemia:

1. Plasma cells must be present in the peripheral blood.
2. Leukocytosis must be present.
3. The bone marrow must be diffusely involved.

A bone marrow biopsy showing diffuse plasma cell infiltration, together with finding the typical cells in the circulating blood, is necessary and usually sufficient.

Bone marrow infiltration by plasma cells unaccompanied by more than occasional plasma cells in the circulating blood may be found in hepatic cirrhosis, agranulocytosis, miliary tuberculosis, carcinomatosis, collagen diseases, allergies, and aplastic anemia. As stated in the discussion of plasma cells, there are a number of blood diseases characterized by transition forms of plasma cells and lymphocytes—lymphoid plasma cells—as seen in Waldenström's macroglobulinemia and in some cases of chronic lymphatic leukemia.

Laboratory findings: In plasma cell leukemia the leukocyte count is usually 10,000-40,000. The percentage of plasma cells found in the peripheral blood varies from 87-1%. The erythrocyte count is low. Anemia follows the same pattern as in other leukemias. There is a low blood platelet count which is reflected in the high percentage of hemorrhagic tendencies. Just as in multiple myeloma, hyperglobulinemia and dysproteinemia are sometimes present in plasma cell leukemia.

Bence Jones bodies: The test for Bence Jones bodies in the urine was performed in 20 of 37 cases of plasma cell leukemia. Six of these were positive, and the rest were negative. A positive test is good evidence of multiple myeloma, for it is infrequent that Bence Jones bodies are found in diseases other than multiple myeloma.

Megakaryocytic leukemia (platelet leukemia)[803, 804]

This is an interesting condition which belongs to the myeloproliferative disorders and which may not be a disease entity *sui generis* but rather a form of myelogenous leukemia, myelofibrosis, or erythroleukemia. The disease probably begins asymptomatically in the early years of life and leads to splenomegaly but is not acompanied by leukemic manifestations. After surgical removal of the spleen, usually because of the discomfort produced by its large size, the blood picture rapidly assumes leukemic characteristics, and evidence of myeloid metaplasia appears in the liver and lymph nodes. Clinically, despite the marked increase in platelets and megakaryocytes, the patient shows bleeding tendencies. The spleen

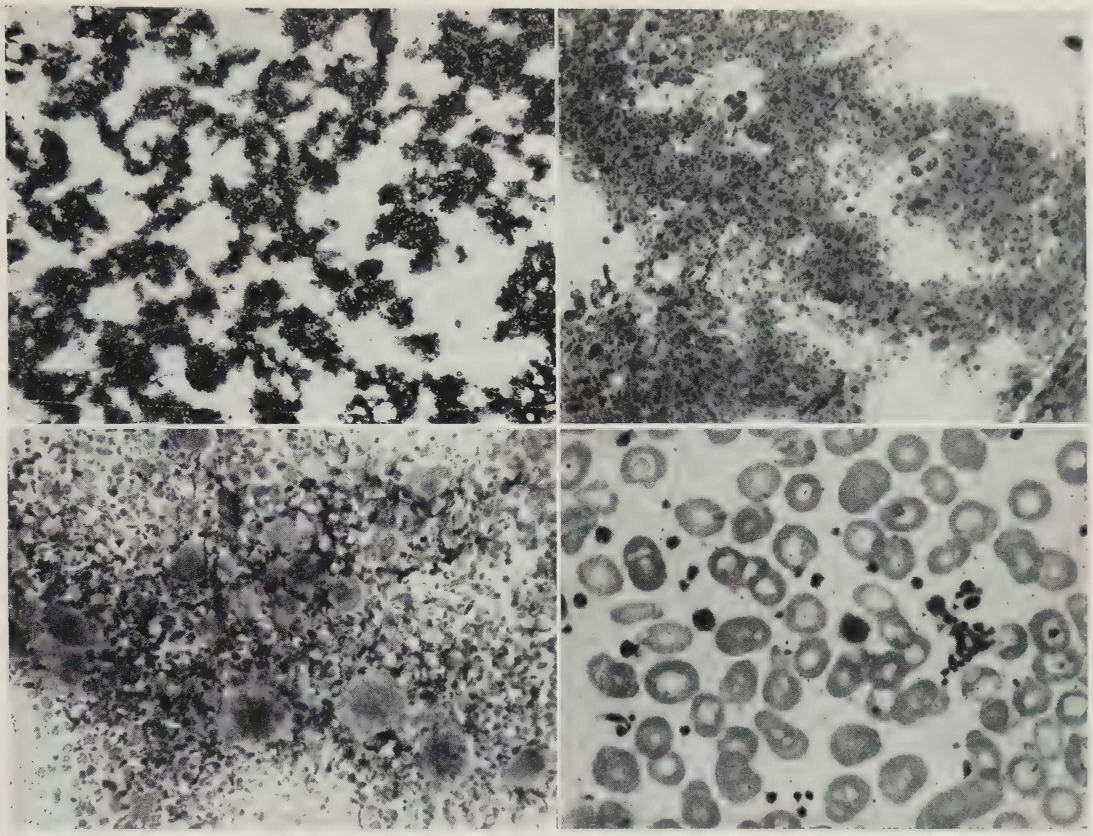


Fig. 450. Megakaryocytic leukemia; bone marrow completely replaced by masses of platelets or by masses of megakaryocytes. Peripheral blood; increased number of abnormal platelets.

appears to be vitally involved in this disease process. Review of the literature shows that not all cases follow the pattern just outlined, but all are preceded by either physiologic removal (fibrosis, calcification, or congenital absence) or surgical removal of the spleen. It thus seems that the loss of the spleen also entails the loss of a bone marrow-inhibiting factor, allowing unrestricted bone marrow proliferation.

Aleukemia, subleukemia, or leukemia without leukocytosis

This disease is so called because of its histologic and pathologic-anatomic symptoms. There are anemia, enlarged glands, and splenic tumor. These cases may show a leukemic blood picture with almost a **normal total count.** Aleukemic conditions may be temporary or may change into manifest leukemia. Pinkus believes that the lymphocytic aleukemia is the most common variety. The blood picture rarely approaches normal. There is relative and sometimes absolute lymphocytosis, with normal or high normal counts. According to Schilling there is a high-grade regenerative shift. There are 60% lymphocytes, with occasional lymphoblasts. These cases are characterized by lymphoblasts, atypical lymphocytes, and Rieder forms with lobulated nuclei, in conjunction with signs of a severe anemia.

Many authoritative sources have reported cases of leukemia without leukocytosis and without enlargement of the spleen. Hirschfeld,[805] for instance, reported 4 cases of leukemia without splenomegaly. Ordway and Gorham[806] reported 1 case, and Kracke and Garver[807] also described 1 case. Weber[808] reported 1 case of aleukemic myelosis without splenic enlargement in which the condition clinically simulated aplastic anemia. He believes that many cases in which the clinical diagnosis was aplastic anemia were undoubtedly examples of aleukemic myelosis. In other words, we can now speak of the condition called **aleukemic myelosis,** which is leukemia without leukocytosis and without enlargement of the spleen. Mettier and Purviance[809] reported 5 cases of leukemia in patients who had no leukocytosis and no splenic enlargements. The clinical findings were those of severe anemia, thrombopenia, and leukopenia with the presence of immature forms of leukocytes in the stained films of blood.

The first case showed 7600 leukocytes/c.mm., with a differential count of 39% segmented cells, 2% nonsegmented cells, 45% lymphocytes, 3% monocytes, 4% myelocytes, 5% myeloblasts, and 2% unclassified immature forms. One nucleated red blood cell was seen per 100 white blood cells. There was diminution in the number of blood platelets.

The patient presented the clinical symptoms of a severe blood dyscrasia and died following hemorrhage from the bladder and gastrointestinal tract. The clinical diagnosis was aleukemic myelosis, with slight leukemic metaplasia of the cervical lymph nodes. Section of this cervical lymph node showed that the architecture of the lymph node had been largely replaced by tumor cells of various sizes, mostly of the myeloid type. There were myelocytic cells of the neutrophilic and eosinophilic types present.

A second case was a 38 yr. old white man who showed 2300 leukocytes or very definite leukopenia, with a differential count of 7% polymorphonuclear cells, 22% nonsegmented neutrophils, 42% lymphocytes, 26% monocytes, 1% myelocytes, and 2% unclassified cells. Two nucleated red blood cells were seen per 100 leukocytes in the stained film. Blood platelets were found greatly reduced. This patient showed anemia and leukopenia, with no apparent hematopoietic reaction to liver extract given either parenterally or by mouth. After a number of weeks the patient returned to the hospital, from which he had been discharged, with the same picture with 20% myelocytes, and 16% myeloblasts, and 40,000 platelets/c.mm. Clinical diagnosis was aleukemic myelosis with anemia and thrombopenia.

There were several other cases reported of similar type.

It is interesting to note that the biopsy of the **bone marrow** from the sternum afforded a valuable aid in establishing the diagnosis. Specimens of the marrow showed a predominance of undifferentiated types of cells, many of which contained mitotic figures packed solidly together to the exclusion of fat cells. This picture of the bone marrow indicated hyperplasia which was undoubtedly leukemic.

DISEASES SIMULATING LEUKEMIA

Lymphosarcoma

Lymphosarcoma is a malignant tumor composed of lymphocytes and lymphoblasts. Hematologically lymphosarcoma may show no detectable changes in the peripheral blood except perhaps a secondary anemia or occasional low-grade lymphocytosis or both. The neoplastic cells may break over into the blood stream in greater or lesser number, producing a type of leukemia first described by Sternberg[810] as leukosarcoma. Wiseman records the results of a series of blood examinations made in a case of lymphosarcoma in which the tumor type of cell (in contrast to normal lymphocytes) was found in the blood stream only intermittently. This case histologically answered the description of the tissue described by Brill et al.[811] under the term follicular lymphoblastoma and is the most chronic type of leukosarcoma encountered.

Chloroleukemia

The term is of historic interest only. Chloroleukemia is a disease described by Naegeli. It displays an acute leukemic blood picture. The symptoms are cachexia, severe anemia, and subperiosteal circumscribed proliferations, manifested principally on the skull. In children it is usually due to an acute myelogenous leukemia.

Leukosarcoma

Leukosarcoma was classified by Sternberg as a separate entity. These cases show the picture of acute leukemia of the lymphocytic type associated with lymphosarcoma (see foregoing discussion of lymphosarcoma).

Pseudoleukemia

The term pseudoleukemia should be discarded. It is of historic importance only and at one time described any disease exhibiting hepatosplenomegaly without a concomitant leukemic blood picture.

Hodgkin's disease

Hodgkin's disease is a malignant tumor of the reticulum cells and lymphocytes. It is of clinical importance in the differential diagnosis of leukemia but has no characteristic blood picture. The peripheral blood may reflect bone marrow involvement (myelophthisic anemia) and hypersplenic activity (hemolytic anemia) and may according to isolated reports very rarely contain Reed-Sternberg cells.

The disease was described by Hodgkin in 1832 in a publication entitled *On some morbid appearances of the absorbent glands and spleen.*[812] Many observations have been made since that time. Considerable experimental work has been done, but the etiologic agent has not yet been discovered. This disease was once called pseudoleukemia. Bonfils in 1856 spoke of it as a cachexia without leukemic changes. Virchow in 1865 classified it with the lymphosarcomata. Cohnheim, unfortunately, introduced the term pseudoleukemia, but this has been proved to be manifestly an incorrect classification. Billroth in 1869 called it malignant lymphoma; Langhans in 1872, malignant lymphosarcoma; Schulz in 1874, reticulated desmoid carcinoma; Orth in 1887, malignant aleukemic lymphadenoma; Langebeck termed it gland sarcoma.

Blood picture: The hematologic picture of this disease has been a subject that has been much discussed. **Certain it is at this time that there is no characteristic picture in the blood by which one can identify the disease as Hodgkin's disease.** There are changes, it is true, but they are not characteristic. The red cells are reduced in number, and there is usually a diminution in the percentage of hemoglobin. The red cell count may vary from 2,000,000-4,000,000 but sometimes drops to the million mark. The red cells are pale, and there are often many microcytes. Occasionally nucleated red cells are seen, together with poikilocytosis, polychromasia, and basophilic punctation. The number of white cells varies. Occasionally Hodgkin's disease displays an unusual increase in the number of white cells, but this increase is not a characteristic phenomenon. There may be leukocytosis as well as leukopenia. McAlpin[813] found the cell count ranged in 18 cases between 2000 and 13,000.

Ziegler[814] in 70 cases found the following: in 22, counts of from 5500-10,000; in 20, from 10,000-20,000; in 8, from 20,000-30,000; in 2, more than 30,000; and in 18, a leukopenia of from 2000-5500. In a general way, there is usually in the early stages of the disease a slightly increased total count or perhaps a normal count.

The differential count has been much discussed, but as yet no one has had the temerity even to suggest that there is any possibility of making a diagnosis of Hodgkin's disease from the differential count. Early in the disease there may be lymphocytosis, but this is not positive. Leukopenia with relative lymphocytosis has been described in the beginning of the disease. Much emphasis has been laid upon the **eosinophilia** in Hodgkin's disease, but this has been denied by others. **The eosinophilia, if present, is of diagnostic importance, but if absent, is not sufficient to exclude this disease.**

The actual diagnosis of Hodgkin's disease can be made only by excision of one of the lymph nodes and histopathologic study.

Nonhematologic findings: In common with the tumors of lymphatic tissue, Hodgkin's disease shows abnormalities of the serum protein characterized by an increase in the alpha globulins and in the component containing ceruloplasmin, whereas albumin and the siderophilin-containing component are decreased.[815]

BRILL-SYMMERS DISEASE (GIANT FOLLICULAR LYMPHOBLASTOMA)

Brill-Symmers disease involves the lymph nodes and the spleen. The lesion is essentially a neoplasm of the reticulum cells. Its importance lies in the fact that it must be considered in the clinical differential diagnosis of the leukemias. The blood picture is not diagnostic.

Clinical symptoms: Lymph nodes become enlarged, remaining discrete, and may become as large as 10 cm. There are usually weakness, anorexia, loss in weight, and low-grade fever.

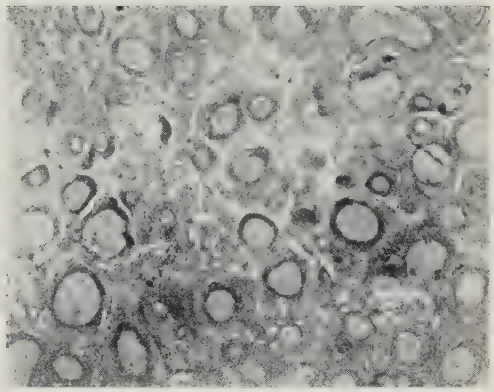


Fig. 451. Benign type of multiple giant follicular lymphoblastoma. (Courtesy Dr. Walter Siebert, St. St. Louis, Mo.)

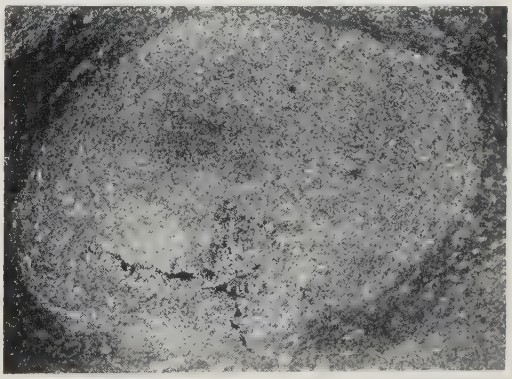


Fig. 452. High-power enlargement of lymphoblastoma. (Courtesy Dr. Walter Siebert, St. Louis, Mo.)

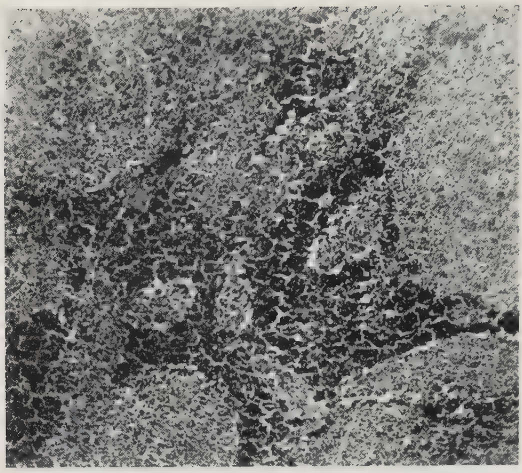


Fig. 453. Low-power view of early transformation of giant follicular blastoma into polymorphous cell lymphosarcoma. (Courtesy Dr. Walter Siebert, St. Louis, Mo.)

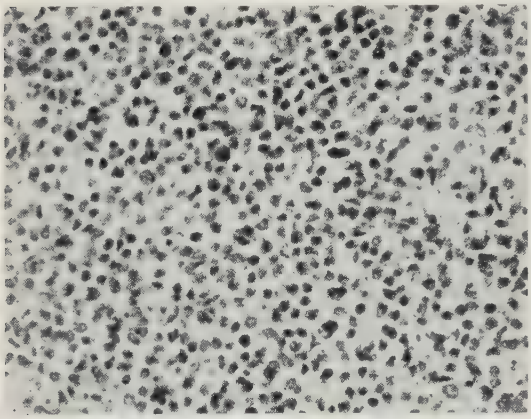


Fig. 454. High-power view of polymorphous cell lymphosarcoma arising from a giant follicular blastoma. (Courtesy Dr. Walter Siebert, St. Louis, Mo.)

Blood picture: Ten per cent of the cases show mild leukopenia, with lymphocytosis of from 50-60%, and mild eosinophilia of 4-6%. There may be a slight anemia and occasionally thrombocytopenia, probably of hypersplenic origin. The bone marrow is usually not involved.

The disease is important because it is likely to be confused with other conditions. Biopsy of the glands should be sufficient to clarify the situation.

LYMPHADENOPATHY

One may divide all lymphadenopathies into 2 classifications: (1) those in which the predominant characteristics are significant of malignancy and (2) those in which the reaction is like an infectious process.

The first classification is composed of 3 clinical subdivisions: (1) lymphosarcoma, including those tissues having the pathology typical of lymphosarcoma, lymphoblastoma, reticulum cell sarcoma, or plasmacytoma, (2) the leukosarcomas and the aleukemias as well as the pure myelocytic, lymphocytic, or monocytic leukemias, and (3) true tumor metastases.

The second major classification, the infectious nonmalignant lymphadenopathies, comprise 4 clinical types: (1) lymphatic tuberculosis, (2) syphilis, (3) simple lymphadenitis (infections of focal origin), and (4) infectious mononucleosis.

Differential diagnosis: At this point the knowledge of the regional anatomy of the lymphatic tissues is indispensable in differential diagnosis; for instance, enlargement of the preauricular node may be due to a focus of chronic conjunctivitis. In order to make a correct diagnosis one must consider the following points: temperature, characteristics of the nodes, splenomegaly, blood picture, and biopsy.

Fever is found only among the diseases of the infectious group.

With respect to the characteristics of the nodes, the presence or absence of pain, the distribution of the swellings, whether local or generalized, and third, the physical characteristics imparted to the palpating fingers by the diseased nodes are points to be determined. Painful glands suggest infection which may be inflammatory.

Distribution of the gland swellings is important. Thus generalized adenopathy without fever is characteristic of the chronic leukemias, the afebrile stage of Hodgkin's disease, and the benign hypertrophies; if fever is present, a generalized swelling means syphilis, Hodgkin's disease, infectious mononucleosis, generalized lymphatic tuberculosis, serum sickness, or an acute phase of leukemia. Local swelling without fever suggests lymphosarcoma, tumor metastasis, lymphadenosis, tertiary syphilis, or Hodgkin's disease. Local swelling combined with pain and inflammation is nearly always indicative of local infection, either lymphatic tuberculosis or pyogenic lymphadenitis, but never of Hodgkin's disease.

Palpation gives important data. Some cases of metastatic carcinoma and some very chronic types of Hodgkin's disease show very hard and unyielding glands, but many do not. Many malignant adenopathies and cases of Hodgkin's disease exhibit discrete not fused glands. In leukemia the glands are always discrete and elastic to the touch. A chronic fluctuant or soft node if painful, adherent to the skin, or showing evidence of previous sinus infection usually means localized lymphocytic tuberculosis.

So far as enlargement of the spleen is concerned, a hard spleen extending nearly to the umbilicus with concomitant generalized adenopathy nearly always means leukemia—and the larger the spleen the more likely is the leukemia to be myelocytic in type. In Hodgkin's disease the spleen does not usually extend much below the costal border and is not palpable at all in one-half of the cases. A slightly enlarged spleen is seen in infectious mononucleosis.

Blood picture: In malignant cervical adenopathies the blood picture is normal except in those patients in an advanced stage or with leukemia. Leukocytosis with neutrophilia is strong evidence that the adenopathy in question is of infectious origin. With lymphocytosis, mononcytosis, and mild anemia of the hemoglobin type, we suspect lymphatic tuberculosis; with lymphopenia, eosinophilia, and monocytosis, and usually leukopenia, with marked anemia of the red blood cell type, the blood reflects Hodgkin's disease. Normal total red and white cell counts, with eosinophilia and lymphocytosis suggest syphilis; marked lymphocytosis and normal red cells suggest infectious mononucleosis.

Biopsy: Select the gland that is most likely to contain the most pathology; i.e., usually the largest gland. Remove the entire tissue but do not cut across or rupture the gland.

TREATMENT OF LEUKEMIA AND MALIGNANT LYMPHOMAS

There is as yet no satisfactory treatment of leukemia or malignant lymphomas, but interest is great as the tremendous amount of basic research and animal experimentation in this field bear witness. Despite the generally poor results in treatment, advances have been made that suggest the possibility of success in the future. Perhaps the most outstanding feature of present-day therapy is the attack on these diseases with the use of chemotherapeutic agents based on some new concepts.[816] Basically this concept is that the cancer cell (leukemic or otherwise) can be regarded in principle much like invading microorganisms. These latter can be destroyed by chemotherapeutic agents while in the human host. Then why not attempt to destroy cancer cells in the same way without injuring normal cells? The basic principles of this approach are (1) the principle of dynamic equilibrium and (2) that of specific nutrition. Principle 1 states fundamentally that all cells require a constant supply of proper nutriments to survive. Principle 2 states that different cell types may have different nutritional requirements either from their external environment or within their internal mechanism. This concept has been referred to as the heterogeneity of nucleic acid anabolism. The researches of the Sloan-Kettering group have been directed at applying these concepts by selective feeding of modified chemical agents to cancer cells effecting a starvation of the cell particularly directed at nucleic acid metabolism. These investigators have repeated in tissue culture of normal and cancer cells what can be shown in bacterial cultures, namely, selective destruction of cells by chemical agents.

SubbaRow's[817] synthesis of folic acid (pteroylglutamic acid) and the finding that the clinical course of acute leukemia was unfavorably affected by this substance or its conjugates led Farber and associates[818] to use folic acid antagonists in the treatment of acute leukemia in children. The Sloan-Kettering investigators have carried on extensive animal research with these chemical compounds, as well as with others, with very promising results. Using 10 different compounds on 5- to 7-day-old tumors in mice, 1275 animals (67%) out of 1910 treated were cured. In mouse leukemia, 44% cures were effected with 6 selected compounds. Amethopterine, an analogue of Aminopterin, was found 98% effective against Line I mouse leukemia but effected no cures in Line I/A, a resistant strain. However, when a combination of P-165 (azaserine or 0-diazoacetyl-L serine) and 6-MP (6-mercaptopurine)* was used against this resistant Line I/A strain, 98% of the mice were cured of leukemia. These results are most encouraging in that an approach appears possible that may shed a ray of hope in the treatment of human leukemias.

To date no human cures of leukemia by chemical or other agents have been effected. The literature on the use of these agents is voluminous and has been excellently reviewed by Wright et al.[819] Therapy with the folic acid antagonists has been the subject of a lengthy supplement of *Blood*.[820] Chemotherapy of leukemia, Hodgkin's disease, and related diseases based on 10 years' experience has been reported by Wintrobe et al.[821] The folic acid antagonists which have been used in the treatment of leukemia are Aminopterin, Amethopterin, Amino-an-fol, Adenopterin, and Aminoteropterin. All of these related compounds are pteroylglutamic or pteroylaspartic acid derivatives. Farber's enthusiasm for the results achieved with these antagonists has not been shared by everyone, and results from their use have varied. Whatever else may be said about these compounds, one thing stands out in our minds: hopeful events have happened in patients with acute leukemia that never happened before these agents came along. Everyone who has treated acute leukemia, especially in children, agrees that the folic acid antagonists have a profound effect on this disease, which in the past left us dismayed and hopeless as it ran its speedy course unchanged by any known form of therapy. The most striking cases are those in which the peripheral blood picture may approach normal and in which the bone marrow shows considerably diminished leukemic activity. It is claimed that in some rare cases autopsy failed to show any evidence of leukemia. Clinically these patients display improved vigor, require fewer transfusions, and are generally useful. These remissions may last only a few days up to many months.

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*Purinethol; Burroughs Wellcome & Co., Tuckahoe, N. Y.

When one tries to analyze statistics as to the benefits derived from folic acid antagonist therapy, there are many variables that readily account for the differences in opinion. Cases of acute leukemia are fortunately relatively rare, and very few observers have had an opportunity to manage sufficiently large enough numbers of patients to make their statistics entirely acceptable. The classification of acute leukemias may vary from one group of investigators to another, which adds another variable to statistical analyses. As with all investigations and therapeutic approaches to disease, the intensity of interest and enthusiasm of the investigator is often reflected in beneficial results in patients not achieved by physicians whose interest is more casual. This involves not only more careful dosage schedules but also care in general management that adds some imponderables to successful therapy.

With folic acid antagonist therapy there is no unanimity of opinion as to whether treatment should be pushed to toxicity with high dosage and a rest period or whether modest dosage schedules should be followed intermittently, or if there should be continuous therapy. Whatever schedule is followed, leukocyte counts and the blood picture are generally helpful and necessary in following these cases. In some cases the white cell count may return to normal or nearly normal values, with disappearance or diminution of leukemic cells in the blood film.

One of the troublesome problems in many of these patients is that leukopenia is a common accompaniment of the natural history of acute leukemia and that the blood films may show relatively few leukemic cells. Studies of the bone marrow in such cases show a wildly active leukemic process. Should these patients be given a leukopenia-producing agent? If so, what should determine dosage? Situations such as these appear to require more courage than scientific knowledge. When toxicity occurs, the symptoms may be more distressing than those related to the leukemia: affection of the gastrointestinal tract, with gingivitis, stomatitis, anorexia, and bloody diarrhea. Folic acid and citrovorum factor, vitamin B_{12}, antibiotics, and liver preparations have been suggested to counteract those toxic effects but have been generally ineffectual. Hemorrhagic diatheses from thrombocytopenia or ulcerations in the gastrointestinal tract present serious problems.

Another complication of therapy is resistance to certain preparations in human beings just as in the mouse experiments previously cited. Such resistance does not militate against use of other agents.

Perhaps the results with the use of folic acid antagonists can be summarized by reiterating that in some patients effects occur that have not been previously observed in acute leukemia. These favorable responses occur much more frequently in children than in adults, and their duration is variable but with no expectancy of cure. Other forms of leukemia are not favorably affected by these agents.

6-MP is an antimetabolite used in the treatment of leukemia. This substance acts as a purine antagonist against certain forms of bacteria, but its mode of action in leukemia is unknown, albeit apparently in a manner different from the folic acid antagonists. Burchenal et al.[822] have summarized their clinical evaluation of this agent in a very comprehensive review including the pharmacology, clinical results, and toxicology together with a series of representative case histories. The results appear to follow those obtained with folic acid antagonists with more strikingly beneficial results in children with acute leukemia, 15 out of 45 such patients showing good clinical and hematologic remissions and another 10 patients showing partial improvement. In adults remissions in acute leukemia have been only occasional, and temporary remissions have occurred in some cases of chronic myelocytic leukemia. The lymphomas, including lymphocytic leukemia and lymphosarcoma, were unaffected by 6-MP. In some instances of acute leukemia in children with resistance to folic acid antagonists or to ACTH or cortisone, 6-MP proved effective in producing some degree of remission. Resistance to 6-MP develops more rapidly than with the folic acid antagonists, but there is apparently no cross resistance between the two. Toxic effects were primarily hemopoietic, with leukopenia and bone marrow depression. However, there is cessation of this process on withdrawal of the drug. Gastrointestinal symptoms were generally mild and not of the order of severity of those produced by the folic acid antagonists. Tivey and Bross[823] summarized the results of a conference on therapy with 6-MP as follows.

Of 337 children with acute leukemia treated in 13 clinics, 36% were improved. The percentage varied from 13 in one clinic to almost 70 in another. Variables such as dosage schedules, terminology, and the use of other agents in conjunction with 6-MP made interpretation of the results difficult.

In this same report there is an illuminating block chart (Table 274) showing that as new compounds become available more children with acute leukemia survive 1 year or longer with treatment. Ordinarily 5% survive beyond 1 year; with Amethopterin and cortisone, approximately 30% carry on for 1 year or longer; with Amethopterin, cortisone, and 6-MP, 50% survive the year.

Urethane (ethyl carbamate) has been found an effective and relatively simple agent in the therapy of chronic myelocytic leukemia and in multiple myeloma. As with all the other chemotherapeutic agents, a host of papers on the subject may be found in both the domestic and foreign literature. It effects a lowering of the leukocyte count in chronic myelocytic leukemia and at times may cause some diminution in organ enlargements in this disease. Its greatest recommendation is the ease of administration (oral) and its relative lack of discomfiture. Toxic symptoms, other than those related to the hemopoietic system, are relatively mild and disappear upon withdrawal of the drug. Rare instances of severe liver damage may occur. Since leukopenia and thrombocytopenia of serious proportions may occur, treatment with urethane should be monitored with frequent observations of the blood picture. When the leukocyte count reaches normal levels, dosage should be diminished or lowered. Our impression is that urethane therapy holds a position in relation to the treatment of chronic myelocytic leukemia that arsenotherapy used to hold.

Nitrogen mustard (methyl-bis [β-chloroethyl] amine hydrochloride) is the mustard gas of World War I vintage and has had its rebirth as a chemotherapeutic agent in the treatment of the malignant lymphomas, being most successful in Hodgkin's disease. The nitrogen mustards are representative of a group of antileukemic agents categorized as radiomimetic. Although the mustards have been tried in many forms of leukemia, their effectiveness has been greatest in Hodgkin's disease, some cases of generalized lymphosarcoma, and perhaps have an occasional worthy application in other selected cases. The mustards are administered intravenously and may cause severe chemical phlebitis or severe reactions at the site of administration if accidentally infiltrated into the tissues. Nausea and vomiting of a disturbing degree are frequent toxic side reactions. There is considerable difference of opinion as to just where to place these agents in relation to roentgen radiation therapy. Generally speaking, there are few if any beneficial effects produced by the mustards that have not been produced by roentgen radiation. Relative costs do not appear a basis for choice. Although nitrogen mustard is less expensive, it is best handled under hospitalized conditions, adding to the cost of therapy. Toxic manifestations such as nausea and vomiting are much more frequent with mustards than with roentgen radiation. Toxic hematologic effects, particularly thrombocytopenia and leukopenia of severe degree, are more likely to occur after therapy with the mustards than with roentgen radiation. In general, when organ enlargements such as localized lymphadenopathy or splenomegaly of considerable proportions are present, direct radiation is much more likely to accomplish shrinkage of these organs than are the mustards. Pruritus, which may be very distressing in Hodgkin's disease, responds favorably more frequently to mustard therapy than to radiation therapy.

It is frequently claimed that when the malignant lymphomas become resistant to roentgen radiation the disease will still react favorably to the mustards and may become radiosensitive again. For this reason alternate courses of therapy with mustards and radiation have been recommended and in some investigations simultaneous therapy with both agents has been tried.

Triethylenemelamine (TEM) was the first of a group of ethylenimine derivatives having nitrogen mustardlike actions. Included in this group are triethylenephosphoramide (TEPA), triethylenethiophosphoramide (thio-TEPA), and diethylenephosphoramide (DEPA). The therapeutic effects with these substances are fairly similar to those seen with the nitrogen mustards, Hodgkin's disease being the most favorably affected, although some control of chronic lymphocytic and myelocytic leukemia has been effected. An advantage over the mustard therapy is that these agents may be given orally. Among the toxic effects, the most significant are related to suppression of hemopoiesis, with leukopenia and thrombocytopenia not uncommon. Cumulative toxic effects may reach serious proportions, and since there is a narrow margin of safety between desired clinical effects and toxic reactions, careful monitoring of the hematologic status of these patients is essential.

Myleran (GT 41) is one of a series of sulphonic acid and other esters which were synthesized,[824] particularly with the idea that they might exert nitrogen mustardlike biologic action. Galton[825] reported on the use of this agent in leukemia and found it effective in producing remissions in chronic myelocytic leukemia. Its major field of usefulness appears to be in this form of leukemia because of its depressive action on the myeloid series. Thrombocytopenia may be an important undesirable toxic effect.

Steroid therapy in the form of cortisone or corticotropin (ACTH) has been used in selected cases of leukemia and malignant lymphomas. One gathers the general impression that these agents are most helpful in those cases which have as an associated phenomenon some secondary immunohematologic features, such as hemolytic anemia, thrombocytopenia, or leukopenia. Consequently, these agents have not been used as primary weapons against such malignant disease but rather as adjunctive therapy along with other therapeutic approaches. Such situations arise frequently in the acute leukemias and in selected cases of chronic leukemias and malignant lymphomas in which splenic involvement is prominent. The incidence and character of toxic effects with ACTH and cortisone are similar to those accompanying their use in other disease processes. These include Cushing's syndrome (acne, hirsutism, edema, moon facies, hypertension, and glycosuria), mental depression, and psychoses.

Radiation therapy "is still the sheet anchor for treating chronic leukemias"[826] and, we might add, the treatment of malignant lymphomas. Radiation has been used in the form of external radiation or internal radiation (radioactive isotopes). Variations in dosage schedules and methods of application are numerous, with champions representing the various schools of thought. As with many other therapeutic procedures, the enthusiasm and persistence of the therapist color his views. The end results of radiation, regardless of method, appear to be about the same insofar as longevity and relief of symptoms are concerned. Toxic symptoms of external radiation include radiation sickness, which is seldom seen with isotope therapy. On the other hand annoying hematologic complications, such as severe leukopenia, anemia, and thrombocytopenia, are more frequent with isotope therapy than with external radiation. The shrinkage of organ enlargements, such as splenomegaly or lymphadenopathy, can be more effectively accomplished with radiation therapy than with isotopes.

The most frequently used isotope is radioactive phosphorus (P^{32}). This agent may be administered orally or intravenously. There is a period of approximately 3-4 wk. before therapeutic response is seen. Colloidal gold (Au^{198}) has been used in some cases of chronic myelocytic leukemia[827] and it has been tried in the treatment of effusions caused by malignant neoplasms.[828]

Radiation therapy is not used in the treatment of the acute leukemias. It has a very useful place in the malignant lymphomas, with external radiation generally favored over isotope therapy.

A host of miscellaneous forms of therapy has been reported with varying results. Myelokentric and lymphokentric acids[829] have been administered on the basis of the reciprocal actions of substances affecting the myelopoietic and lymphopoietic systems. Vitamin B_{12} and other hematinics have been generally found ineffective. In addition, a large number of chemicals known to produce leukopenia in animals and human beings have been given limited therapeutic trials.

Transfusions play a very important role in the management of the anemia associated with leukemia and malignant lymphomas. The ready availability of blood has been a major factor in the rehabilitation of many of these patients whose anemia generally does not respond to oral hematinics unless there is an associated blood loss. It is our opinion that many of the benefits of modern care of these patients can be attributed to the free use of whole blood.

Almost all of the antibiotics have been used as adjunctive therapy in the treatment of the leukemias. The display of these agents has embodied both prophylactic therapy and the treatment of actual infection. As one reads some of the literature, there is an overtone suggesting that the antibiotics might have some beneficial influence on the leukemic process other than those effects related to infection. Our experience is that in the absence of infection the antibiotics can play only a questionable role.

With the growing consciousness of immunohematologic mechanisms, more and more patients with leukemia and lymphomas are being considered for splenectomy. The patients with secondary hemolytic anemia are the ones in whom splenectomy has been frequently considered, especially in chronic lymphocytic leukemia. Poor results with splenectomy have been recently reported in 5 advanced cases of Hodgkin's disease.[830] It would appear that unless well-proved immunohematologic mechanisms can be demonstrated in an individual patient, splenectomy should not be considered. Splenectomy, when effective, affects only the abnormal immunologic process, and whatever benefits accrue to the patient are the result of such influences. The

Table 274. *Chemotherapeutic agents useful in treatment of neoplastic diseases**

Agent	*Average dose*	*Diseases benefited*	*Effect of overdosage: complications*
Urethane	0.5-1.0 gm.	Multiple myeloma Myeloid proliferative diseases Leukemia, chronic granulocytic Polycythemia vera?	Anorexia, nausea, vomiting, olfactory disturbance Leukopenia, pancytopenia (rare) Centrilobular hepatic degeneration and necrosis
Nitrogen mustard (HN_2)	0.1 mg./kg. I.V. 4-6 times	Lymphoid proliferative diseases Hodgkin's disease (generalized) Lymphomas, malignant Some anaplastic carcinomas	Acute nausea, vomiting Pancytopenia Bone marrow depression (temporary)
Triethylenemelamine (TEM)	2.5 mg., fasting with 2 gm. soda/day 2-3 times initially; 1-5 mg./5-10 day as maintenance	Chronic lymphocytic leukemia Lymphocytic lymphoma Hodgkin's disease (generalized) Chronic granulocytic leukemia Polycythemia vera Adenocarcinoma ovary	Anorexia, nausea, vomiting Leukopenia, severe Pancytopenia
ACTH	20 units I.V. for 15-18 hr./day, or 20-40 units gel I.M./day	Acute leukemia Chronic lymphocytic leukemia with marrow damage Hemopoietic malignancies with symptomatic hemolytic anemia	Edema Cushing's syndrome: moon facies, acne, hirsutism, edema, hypertension, hyperglycemia (glycosuria) Hypokalemic, hypochloremic alkalosis Mental depression, psychoses
Cortisone	25-50 mg. q 4-6 hr.	Same as ACTH	Same as ACTH
Folic acid antagonists	Aminopterin, 0.5-1 mg., orally, 1-3 times/week	Acute leukemia	Pancytopenia Mouth ulceration Diarrhea
6-Mercaptopurine (Purinethol)†	1-2.5 mg./kg., daily initially	Acute leukemia Chronic granulocytic leukemia	Leukopenia Pancytopenia
Myleran†	6 mg./day, orally initially; 2-4 mg./day maintenance	Chronic granulocytic leukemia	Leukopenia Pancytopenia

*Revised from Rundles, R. W., Barton, W. B., and Coonrad, E. V.: South. M. J. 46:259, 1953.

†Rundles, Wayne: Personal communication.

basic disease is usually unaffected, or the clinical course may be precipitated into a more rapid tempo.

In the choice of treatment of any patient a host of factors must be given consideration. Undoubtedly the philosophy of the therapist plays a leading role in such decisions. The utter pessimism of some physicians is understandable in the light of the death sentence imposed by a diagnosis such as leukemia or malignant lymphoma. The outlook has been brightened by the well-established effects of the antimetabolites in acute leukemia, in which events have taken place that were never seen before. These results have modified the views of many in the direction of achieving control of these diseases in somewhat the same manner as diabetes is controlled. The approach to such control in our present state of knowledge is limited to a great extent by the untoward reactions of the agents used in combating these diseases. There are many times when the natural course of the disease is better left undisturbed than overzealously treated to a point at which the discomfort from the therapy far outshadows that associated with the disease process. This implies a thorough knowledge of the natural history of the disease, which may be difficult to prophesy in individual cases but has been fairly well categorized in general.

Many studies on life expectancy have been published; there is no conclusive proof that life expectancy is prolonged by our present-day treatment, except perhaps in acute leukemia, especially in children. The pessimistic school has raised the question of whether such increases in prolongation of life as may occur are even worthwhile and point to the added mental anguish and economic strain imposed upon patients and family as sufficient reason to question the wisdom of this prolongation. Social conscience and philosophy become so inextricably enmeshed in these discussions that at times we lose sight of our position. The immature press releases of the optimistic school are as much to be abhorred as is the nihilistic approach of their opposites. There is undoubtedly a middle ground of approach in which the comfort and usefulness of the patient should be our present target of therapy. Judicious application of present technics can accomplish these aims in many cases. In the meantime the genius that will effect real cure has not been revealed to us. The furious attack on nucleic acid metabolism may or may not be the proper answer but indicates an attitude of refusal to be stifled by traditional concepts. Along with this attitude comes a ray of hopefulness that satisfactory answers may come in the not too distant future. Whence will come the prophets carrying the glad tidings remains to be seen.

Chapter 67

Bone marrow studies

JOHN D. BAUER

In all uncertain cases of blood diagnosis it is well to obtain some of the bone marrow for diagnostic examination.

In the adult human being the bone marrow of the long bones is yellow fat; the marrow is red (hematopoietic) **at birth.** In the early years, say about **7 yr.**, fat in small quantities appears; between **12** and **14 yr.**, a visible patch of yellow appears in the middle of the femur which increases until there is only a small patch of red marrow near the end of the diaphysis in the adult. The same phenomenon takes place in the tibia, ulna, radius, humerus, and fibula. The only red marrow which persists in the adult is to be found in the bones of the skull, ribs, vertebrae, sternum, and the innominate bones. Bone marrow studies, therefore, are most conveniently made from the adult sternum.

The bone marrow in infants shows many immature cells. In the **6 mo. fetus** the red cells make up three-quarters of the total; **at 8 mo.**, nine-tenths; and **at term**, the red and white cells are equal. Latimer and Mayerhoff found fetal marrow containing 2-22% granulocytes. Doan and Zerfas state that during the first year of life the marrow contains 4-20% lymphocytes. In embryonic life the ratio of erythroid to myeloid elements is 100:1. In adults, it is 1:3. Since the red bone marrow of newborn children contains many more immature cells than adult bone marrow, it is easy to understand the ready appearance of immature cells in the circulating blood of an infant following slight stimuli. In children, the number of cells in the bone marrow varies from 270,000-1,568,000/ml. In adults we find that in the total bone marrow volume of 1500 ml. in the human body the myeloid elements outnumber the erythroid elements 3:1; most of the myeloid cells, 85%, are the myelocytic cells with their full quota of granules.

There is a diurnal variation in leukocytes with an afternoon and a midnight rise. The bone marrow delivers granulocytes, myelocytes, juvenile cells, and stab cells hourly, but especially at the interval at which the leukocytes in the blood stream are increased. The maturation time from myelocytes to more mature forms is probably from 45 min. to 2½ hr.

One per cent of the bone marrow elements are megakaryocytes. According to Wright these constitute the origin of blood platelets. In bone marrow, 70% of the total erythroid cells are normoblasts; erythroblasts, 26-30%; proerythroblasts, 1-4; and megaloblasts, 0.01-0.04%. According to Schilling the megaloblasts represent a stage in the development of the red cells, but according to Ferrata and Michaelson, the megaloblast is present in the bone marrow only in pernicious anemia. They do state, however, that the cell which is sometimes mistaken for the megaloblast is in reality the proerythroblast. Sabin and Doan corroborate Schilling's claim that the megaloblast is present in normal bone marrow but only in very small numbers.

Reticulated red cells and normoblasts are released into the blood stream at regular intervals; the red cell count varies about 1,000,000/c.mm./day. The high point of bone marrow delivery is in the morning and afternoon, not afternoon and midnight, as is the case with the white blood cells.

SUMMARY OF NORMAL BONE MARROW FINDINGS

What is the normal cellular content of the bone marrow? We must assume, first, that under normal conditions myelopoiesis and erythropoiesis are confined to the bone marrow. Lymphocytopoiesis normally does not seem to be confined to the bone marrow. The investigations of Askanazy, Oehme, von Fischer, and others showed that there are fully developed lymph follicles in the bone marrow in a large number of cases. Under normal conditions, however, lymphocytopoiesis seems to have no connection with either erythropoiesis or myelopoiesis although there is no objective hematologic proof of this. The Schilling followers do not believe that monocytopoiesis is connected with bone marrow function. Monocytes are seen in very few numbers in bone marrow according to Schilling, Sabin, and others. Yamamoto and Schilling[831] have shown in man and in animals that the composition of different parts of the marrow is practically the same on the same occasion in the same individual. In a few cases peripheral eosinophilia occurred which was accompanied by an increase in the proeosinophils in the bone marrow. On the other hand abnormally low numbers of peripheral eosinophils were not represented by low values in the marrow. According to these authors parallelism does not exist between peripheral and bone marrow eosinophilia. Homma[832] found constant bone marrow eosinophilia with an elevation in the number of peripheral eosinophils. But conversely there may be normal or somewhat increased numbers of eosinophils in the bone marrow in spite of decreased number of peripheral eosinophils. Magne is substantially in accord with Homma. Barta found that the effect of toxin on eosinophils is exerted on the fully developed cells which have wandered out and not on the young forms in the marrow which explains the decreased number of peripheral eosinophils without a corresponding decrease in the eosinophils of the bone marrow. Table 275 gives the composition of bone marrow according to a number of authorities.

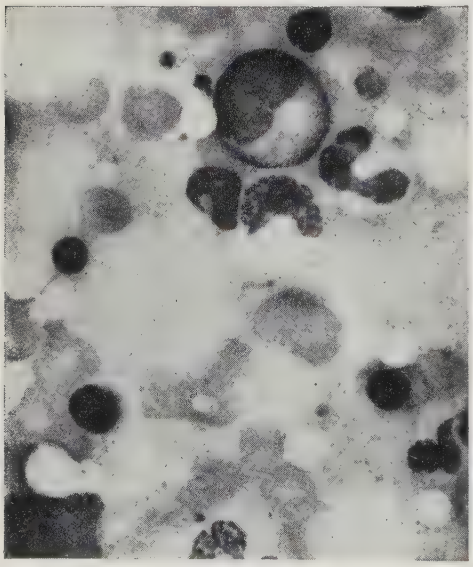


Fig. 455. Normal bone marrow. Note lack of crowding of cells and presence of fat. (×950.)

Holmes and Broun[833] call attention to the mitotic divisions of cells in the bone marrow spreads made by them as being limited to an intensely basophilic cell which varies in morphology from both the typical myeloblast and the typical megaloblast. They have called this cell the primitive cell in their differential counts. They occasionally noted mitotic division in hemoglobin-containing erythroblasts. **Differential counts** by these writers **of bone marrow elements** in 7 cases with normal peripheral blood pictures were as follows: myeloblasts, 2.4%; myelocytes, 7%; juvenile cells, 6.7%; stab cells, 14%; II segmented neutrophils, 10%; III segmented neutrophils, 6.3%; IV, V, etc., segmented neutrophils, 1.1%; eosinophils, 1%; basophils, 0.3%; monocytes and reticuloendothelial elements, 9%; lymphoid cells, 24.9%; megaloblasts, 5.2%; normoblasts, 6.9%; primitive cells, 2.6%. These findings agree rather closely with those of Arinkin[834] on similar types of cases.

It is interesting to note that the high percentage of immature granulocytes in their counts confirms the concept of Arneth and Schilling that the neutrophilic nucleus changes progressively with age from the round to the segmented form. The high percentage of juvenile and stab cells confirms the idea that these are young granulocytes.

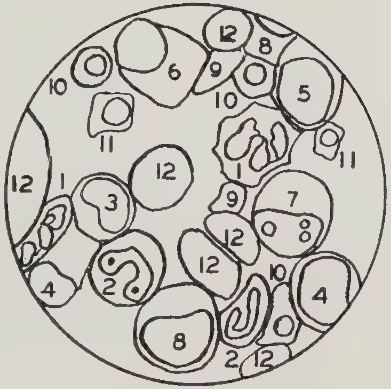
An examination of Table 274 and similar tables in other publications reveals wide variations in opinions on the relative percentages of various cells, and raises some question as to the value of doing differential counts routinely on bone marrows. We are inclined to agree with Israels[835]: "The variability of the normal bone marrow means, in effect, that only gross differences can be taken to indicate pathological change, and this in turn raises a question of whether it is worthwhile

Text continued on p. 1390.

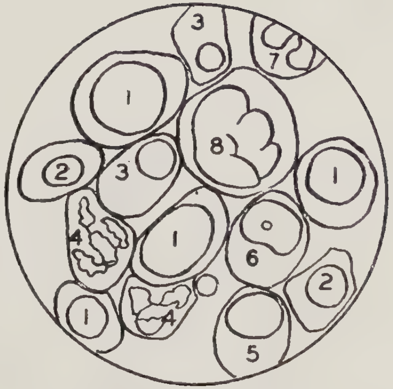
Table 275. *Bone marrow content of normal individuals according to various authors**

		Doan and Zerfas	*Zadek*	*Yamamoto*	*Schilling and Bentzler*	*Varela*	*Barta*	*Weiner and Kaznelson*	*Arinkin*	*Tempka and Braun*	*Nordenson (present material)*
Myeloblasts		1.1-11.9	8	2	–	5.54	2-3	4.6	1.0- 2.4	4.66- 7.0	0.25- 5.50
Metamyeloblasts		–	–	–	–	–	–	–	–	1.50- 5.50	–
Promyelocytes	n	–	14	7	–	8.4	6-8	5	1.0- 2.8	3.75- 6.83	1.25- 8.25
	eo	–	–	–	–	0.57	–	–	–	0.55- 0.66	–
Myelocytes	n	18.8-54.9	25	44.5	34.8-47.4	20.55	40-42	19.4	4.5- 8.6	12.75-13.33	4.25-18.0
	eo	0.1- 4.1	18	4.5	–	0.94	–	1	0.3- 1.0	1.50- 2.66	0- 6.25
	b	0- 0.1	–	–	–	0.22	–	0.2	–	0- 0.33	0- 0.50
Metamyelocytes	n	–	22	22.5	34.0-56.2	27.43	20-22	15.7	1.4- 3.4	14.33-16.25	12.50-42.50
	eo	–	–	–	–	0.73	–	–	0.3- 1.0	0.33- 3.66	–
	b	–	–	–	–	–	–	–	–	0- 0.16	–
Rod forms	n	–	–	9	0-1	–	6-8	63	–	17.0 -22.50	2.25-10.75
	eo	–	–	–	–	–	–	–	–	0.50- 1.0	–
	b	–	–	–	–	–	–	–	–	0- 0.16	–
Segmented forms	n	10.5-55.4	6	8.5	7.0-22.0	–	8-10	22.3	41.5-55.0	16.16-20.33	14.25-35.0
	eo	0- 0.6	–	1	1.1- 4.6	–	–	2.1	0.6- 4.0	0.66- 2.50	0.25- 7.50
	b	0- 0.1	–	–	–	–	–	0.8	0- 0.7	0.16- 0.33	0- 0.75
Lymphocytes		10.8-37.3	7	–	–	–	–	13.6	7.3-16.5	2.66- 3.25	7.5 -38.0
Monocytes		0.5- 4.5	–	–	–	–	–	6.5	2.1- 9.3	0.50- 0.75	0- 5
Plasma cells		–	–	–	–	–	–	2.1	0.3- 0.9	0.25- 1.66	0- 3.25
Clasmatocytes		0.1- 0.27	–	–	–	–	–	–	–	–	–
Unclassified		0.3- 1.1	–	–	11.8-24.8	–	–	–	–	0-3	0- 4.50
Megakaryocytes and megakaryoblasts		0- 0.45	Numerous	–	–	0.29	–	0.2	0.6- 6.1	2.16-4	0- 1
Primitive cells		0.4- 1.1	–	–	–	–	–	–	–	–	–
Ferrata cells		–	–	–	–	–	–	–	–	1.33- 3.75	3-40
Endothelial cells		–	–	–	–	–	–	0.2	–	–	–
Histiocytes		–	–	–	–	0.86	–	–	–	0.50- 1.0	–
Hemocytoblasts		–	–	–	–	2.23	–	–	–	–	–
Megaloblasts		0- 0.1	–	–	–	–	–	–	–	–	–
Pronormoblasts		–	–	–	–	–	–	–	0.8- 2.9	–	0- 6
Basophilic normoblasts		–	–	–	–	5.53	–	–	–	–	1-16
Normoblasts		4.1-13	Numerous	–	31.6-43.7	21.71	–	25.6	5.7-16.0	–	26-184

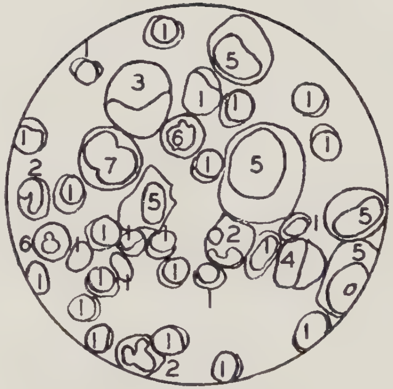
*From Nordenson, N. G.: Studies on bone marrow from sternal puncture, Stockholm, 1935, Generalstabens Litografiska Anstalts Förlag.



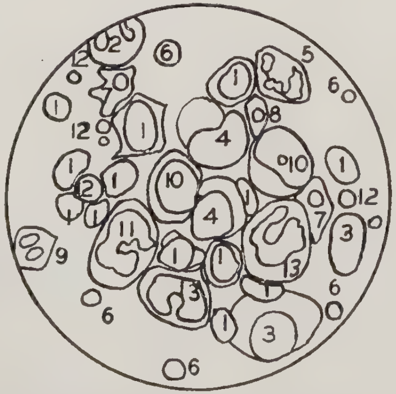
Normal
(Wright-Giemsa stain; ×950)



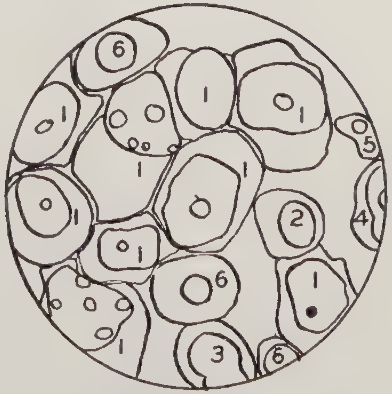
Pernicious anemia
(Wright-Giemsa stain; ×950)



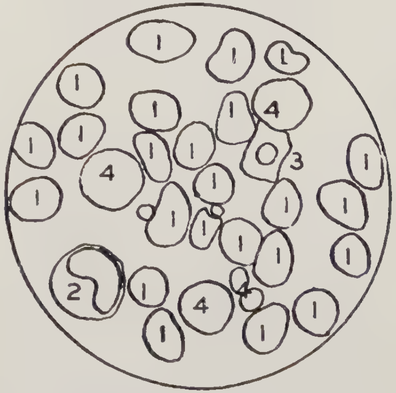
Aplastic anemia
(Wright-Giemsa stain; ×950)



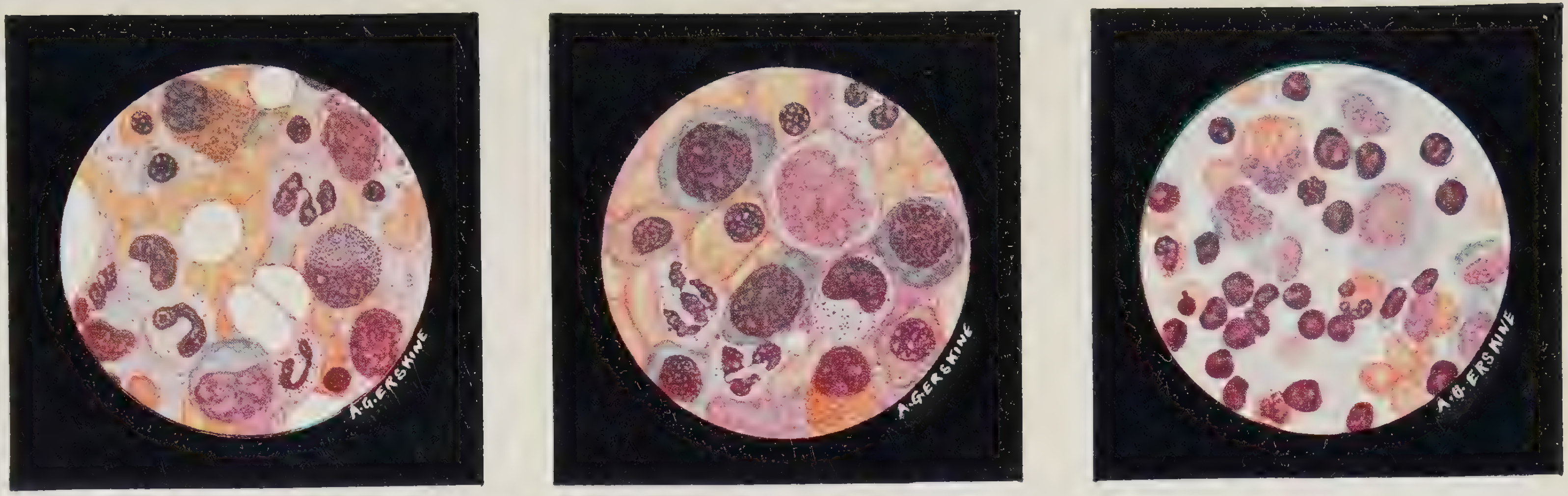
Carcinoma of the liver
(Wright-Giemsa stain; ×950)



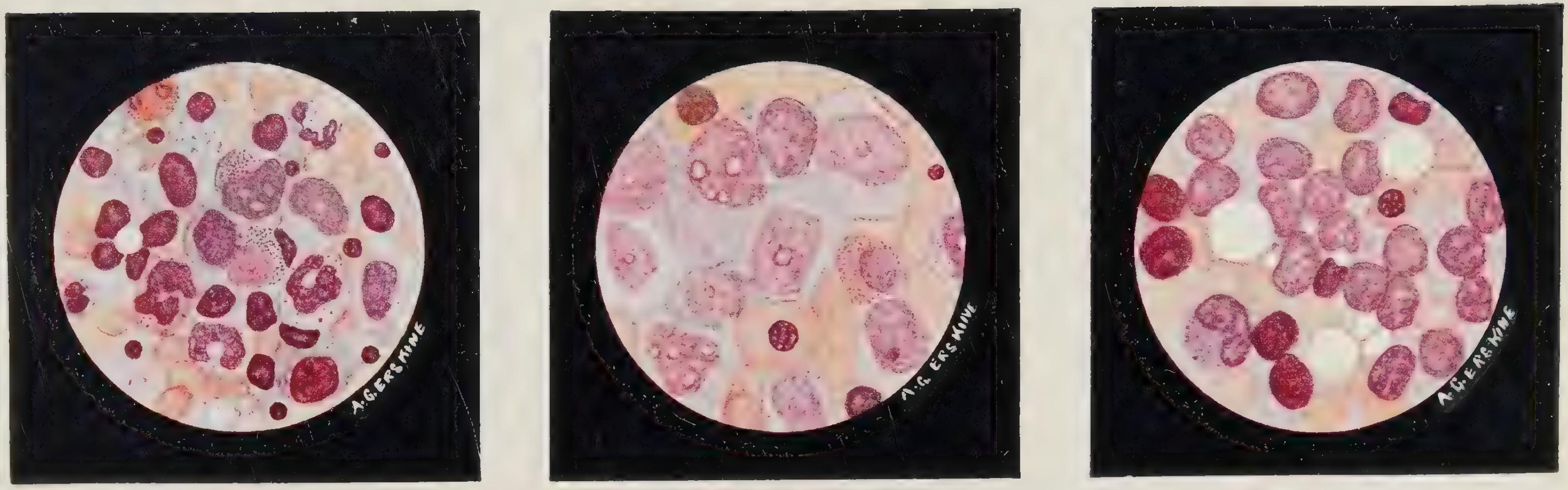
Myelocytic leukemia
(Wright-Giemsa stain; ×950)



Lymphocytic leukemia
(Wright-Giemsa stain; ×950)


A.G. ERSKINE
A.G. ERSKINE
A.G. ERSKINE

Normal **Pernicious anemia** **Aplastic anemia**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A.G. ERSKINE

Carcinoma of the liver **Myelocytic leukemia** **Lymphocytic leukemia**

Fig. 456. Bone marrow touch preparations. For explanation of key see p. 1389.

Fig. 456. Bone marrow touch preparations—cont'd.

Normal

1. Segmented neutrophil
2. Stab cell
3. Neutrophilic juvenile cell
4. Neutrophilic myelocyte
5. Basophilic myelocyte
6. Eosinophilic myelocyte
7. Promyelocyte
8. Myeloblast
9. Polychromatic erythrocyte
10. Orthochromatic normoblast
11. Polychromatic normoblast
12. Fat cell

Pernicious anemia

1. Polychromatic megaloblast
2. Polychromatic macroblast
3. Orthochromatic macroblast
4. Segmented neutrophil
5. Eosinophilic myelocyte
6. Neutrophilic myelocyte
7. Stab cell
8. Atypical cell young giant neutrophilic cell

Aplastic anemia

1. Small lymphocyte
2. Eosinophil
3. Eosinophilic juvenile cell
4. Eosinophilic myelocyte
5. Myeloblast or lymphoblast (?)
6. Orthochromatic normoblast with karyorrhexis
7. Young lymphocyte

Carcinoma of the liver

1. Small lymphocyte
2. Eosinophil
3. Young cells, probably myeloblasts
4. Promyelocyte
5. Segmented neutrophil
6. Nuclei—probably of normoblasts
7. Orthochromatic normoblast
8. Polychromatic normoblast
9. Polychromatic normoblast with karyorrhexis
10. Myeloblasts
11. Basophil
12. Fat
13. Neutrophilic juveniles

Myelocytic leukemia

1. Myeloblast
2. Eosinophilic myelocyte
3. Neutrophilic myelocyte
4. Stab cell
5. Orthochromatic normoblast
6. Orthochromatic macroblast

Lymphocytic leukemia

1. Lymphocyte
2. Neutrophilic juvenile
3. Orthochromatic normoblast
4. Fat

Table 276. *General comparison of spreads and sections of bone marrow*

	Spreads	*Sections*
Cellularity	Varies with technic	Consistent with true state of marrow
Relative distribution of cell species	Irregularities dependent on technic	Accurate reflection
Identification of individual cells	Better than in sections	More difficult than in spreads
Mitotic activity	Morphology of individual mitotic figures better than in sections	Quantitative aspect more readily apparent than in spreads
Histologic structure	Only implied	Readily discerned
Cytochemical studies	Material unsatisfactory	Material satisfactory
Lymphomas	Easily missed or not apparent	Readily identified
Granuloma lesions	Not seen	Readily identified
Malignancy	Individual cells or groups of cells irregularly distributed	Syncytial masses readily found; histologic arrangement may indicate source

to undertake, in every case, the time-consuming differential count . . . this practice has now been given up for a 'qualitative assessment' in all except the most difficult cases, or in research studies. Such a qualitative assessment is adequate for diagnosis in most instances, and gives a far clearer idea of the over-all marrow picture than does a row of figures, especially since few physicians or pathologists carry the normal figures around with them."* We have used an assessment type of reporting clinical marrow aspirations and include the following data in such reports:

Cellularity: Normal, increased, or decreased.

Predominant cell type: Normally this should be members of the myelocytic series.

Myelocytic series: Normally shows predominance of older forms, e.g., stab and segmented cells, with a gradual diminution to myeloblasts. If a left shift is present, comment is made on the degree. Qualitative changes in the cells of this series are described if present. Such changes include toxic granules and abnormal forms. The myeloid-erythroid ratio is estimated.

Erythrocytic series: Normally shows predominance of older forms, e.g., orthochromic normoblasts with a scattering of younger forms. If a shift has occurred to younger members of this series, its degree is appraised and reported. The type of erythropoiesis is described, e.g., definitive (as in normal red blood cell development) or primitive (as in pernicious anemia and some other markedly pathologic states). Qualitative changes indicating pathologic erythropoiesis are noted. These include marked poikilocytosis (poikilocytes, spherocytes, target cells), anisocytosis, basophilic stippling, abnormalities of nuclear morphology (karyorrhexis, karyolysis), and abnormal relationships of nucleus to cell size.

Lymphocytic series: Normally less than 10%. Quantitative or qualitative changes in these cells are noted.

Megakaryocytic series: Normally mature megakaryocytes are readily found, especially in the thin and thick ends of spreads. They tend to be fewer in the middle of spreads. Qualitative changes such as cytolysis with loss of platelet formation are described.

Other cells, such as fat cells, reticuloendothelial cells, and "cells foreign to normal bone marrow (tumor cells)" are noted.

Mitoses are noted as to frequency, and comment is made as to whether mitotic figures are normal or abnormal in appearance.

Such appraisals are not based on spreads alone. Agress[836] has shown the value of studying sections and spreads simultaneously in order to obtain a true quantitative evaluation of bone marrow. A general comparison of the two methods is shown in Table 276 by Agress, which is based on his study of 1187 cases in which bone marrow aspirations were performed.

As can be readily noted from Table 276, many differences are apparent between spreads and sections. Perhaps the outstanding difference relates to quantitative appraisal.

It is not unusual to find spreads which appear extremely hypocellular, whereas the sections may appear quite normally cellular. On the other hand, individual cell morphology is more clearly studied in well-prepared spreads.

In this study a correlation between spreads and sections of bone marrow was set up by Agress in relation to their being diagnostic. Table 277 represents this correlation.

There is close correlation between spreads and sections insofar as the over-all percentage in satisfactory preparations is concerned. However, in individual cases the spread may be unsatisfactory and the section satisfactory. In other instances, the reverse may be true. This correlation is particularly important in the hypoplastic or aplastic states. Note that no cases of aplastic anemia were diagnosed on spreads alone and only 20% were diagnosed on sections of the clot obtained by routine

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*From Israels, M. C. G.: An atlas of bone marrow pathology, New York, 1948, Grune & Stratton, Inc.

Table 277. *Correlation of preparations of bone marrow*

	Spreads (%)	*Sections (%)*
Per cent satisfactory preparations	92	95
Anemia, etiology variable	0*	8*
Aplastic anemia	0*	20*
Carcinoma	90*	100*
Granuloma lesions	0*	100*
Histiocytes	100	0
Hodgkin's disease	3*	8.7*
Leukemia	100*	100*
Leukosarcoma	60*	90*
Lymphosarcoma	9.4*	100*
Multiple myeloma	72*	97*
Myelofibrosis	0*	33*
Pernicious anemia	95*	94*

*Diagnostic.

marrow aspirations. The reason for this should be obvious. In those cases of aplastic anemia in which the bone marrow is almost completely replaced by fibrous tissue, routine marrow aspirations are generally doomed to failure. This diagnosis should not be made on the **absence** of cells from the spread **alone.** If sections of clot show no marrow units, the diagnosis still should not be made since the result may be purely technical in origin. In such an event, the marrow aspiration must be repeated or surgical trephine should be performed to obtain a truly representative specimen of bone marrow. This figure also gives some suggestion of what the physician might expect diagnostically from bone marrow aspiration.

Like all other laboratory examinations, bone marrow studies have their limitations and serve their most useful purpose only when correlated with the clinical aspects of the patient. Bone marrow aspiration should not be performed prior to a thorough history, physical examination, and at least routine laboratory studies. This is particularly true in the vast majority of anemias, except pernicious anemia and a few others. In relation to pernicious anemia and related macrocytic anemias, it is important to remember that erythrocyte maturation factor in even modest dosages will convert the bone marrow picture considerably. For this reason, folic acid, liver, and vitamin B_{12} should not be administered prior to marrow aspiration. In Table 278 by Agress is given an outline of the indications for marrow aspirations. The cases listed under hypersplenism[837] are particularly to be stressed since these are conditions in which splenectomy may exert a very favorable influence.[837] It is well to remember that selected cases displaying the features of hypersplenism may be primarily due to metastatic carcinoma of the bone marrow. Splenectomy could serve no useful purpose in such cases. For this reason, bone marrow aspiration should be performed in all cases in which splenectomy is contemplated as a form of treatment for a blood dyscrasia. It has been our experience generally that the best results in these conditions come in those patients whose bone marrow contains a normally active or hyperactive picture of the blood element or elements affected. For example, we do not recommend splenectomy in idiopathic thrombocytopenic purpura unless the bone marrow shows a fairly good representation of megakaryocytes. Using this criterion alone our results with splenectomy have been exceedingly favorable. Much has been written about the bone marrow picture in idiopathic thrombocytopenic purpura as related to splenectomy, and there is no evidence other than the presence of adequate numbers of megakaryocytes (mature or immature) upon which the many observers will agree.

The second group of conditions with a bizarre blood picture forms a rare heterogeneous group that covers the horizon of medicine. It includes individual cases of drug intoxication, familial cell defects, or the unusual reflection of some well-known disease. It may include leukopenias or hyperleukocytosis as well as unusual cells in the peripheral blood. Abnormal forms of erythrocytes as well

Table 278. *Indications for bone marrow aspiration*

I. Every case of hypersplenism
    A. Thrombopenia
    B. Hemolytic anemia
    C. Leukopenia
    D. Emphatically when A, B, and C are combined (Splenectomy should not be performed in these cases without examination of bone marrow)
II. All cases with a persistent bizarre blood picture
III. Cases in which there is a high index of suspicion of marrow invasive disease, e.g., carcinoma, myeloma, Gaucher's disease, etc.
IV. Corroboration of a limited number of cases of leukemia in which peripheral blood studies are inconclusive
V. Macrocytic anemias of all types prior to therapy with erythrocyte maturation factor
VI. Malignant lymphomas in which lymph nodes are either unavailable or inconclusive
VII. A limited number of infections, e.g., brucellosis, histoplasmosis, tuberculosis, malaria (very rarely), leishmaniasis
VIII. All primary refractory anemias (repeated dry taps may require trephine)

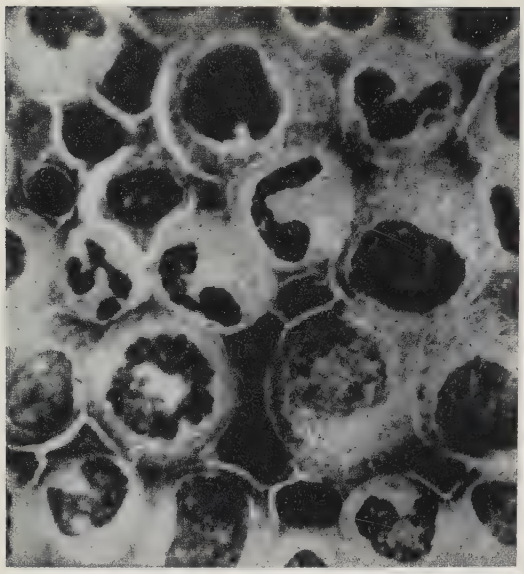

Fig. 457. Infection, bone marrow, showing crowding of cells and absence of fat. (×950.)

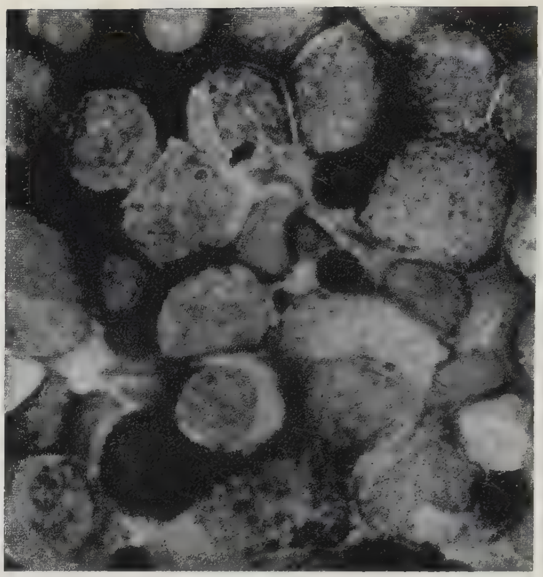

Fig. 458. Hyperplastic bone marrow. Note crowding of cells, marked increase of very young white cells, and absence of fat. (×950.)

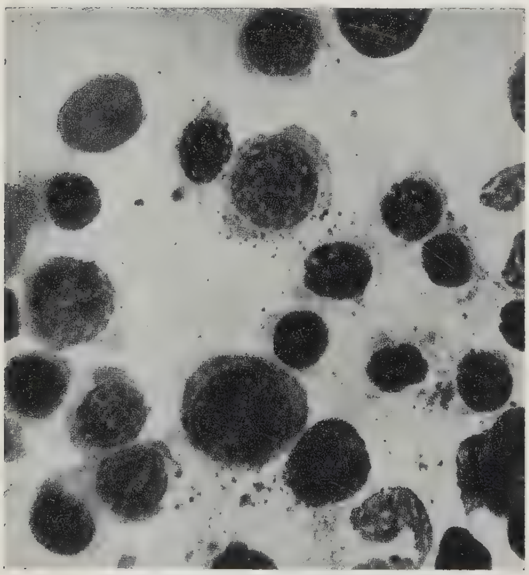

Fig. 459. Bone marrow, erythropoiesis, showing abnormal number of erythroblasts. (×950.)

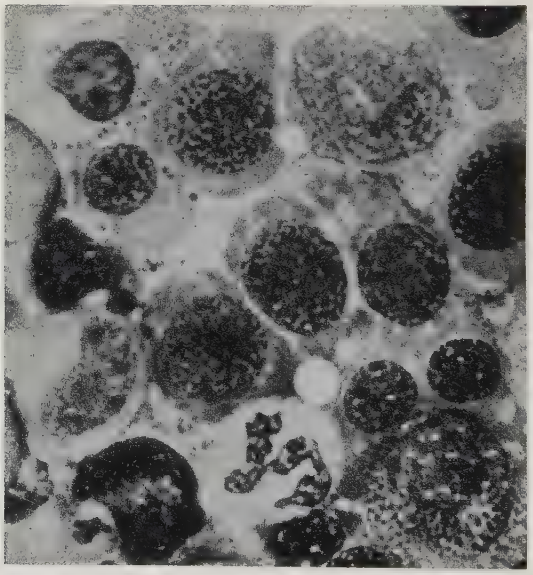

Fig. 460. Pernicious anemia, bone marrow, showing abnormal number of megaloblasts. (×950.)

as unexplained anemias should be included here. Thrombocytopenias or morphologically abnormal platelets (giant platelets) are included. Many of these cases are associated with organ enlargements such as the spleen and liver, suggesting some blood dyscrasias. Some of these cases are examples of hypersplenism, and as our knowledge of immunohematology broadens, they are being more accurately categorized. Some of these cases are in reality malignancies, with or without demonstrable bone marrow metastases.

The diagnosis of bone marrow invasion by metastatic carcinoma is dependent upon a number of factors, most important of which is a high index of suspicion. In our experience, the finding of **one or more normoblasts in the peripheral blood of an adult not having an obvious disease explaining the presence of normoblasts** is one of the most important **indicators of possible malignant invasion** of the bone marrow. Not all cases of bone marrow malignancy show normoblasts in the peripheral blood, but when normoblasts are present, we pursue the possibility of malignancy vigorously. Agress uncovered 2 cases of unexpected malignancy with normal blood values in which normoblasts were found on routine blood spreads. The success with which marrow aspiration will reveal metastases is dependent upon the vigor with which such searches are made. If multiple sites are aspirated and numerous slides are examined, the positive results may be very high. Sections of bone marrow clot often will show tumor cells otherwise difficult to find in spreads alone. The section technic outlined by Agress may also give some information as to the original site of the malignancy by the histologic structure assumed by the tumor cells.

MULTIPLE MYELOMA

Multiple myeloma is a form of malignant tumor occurring in the skeletal trunk of adults and infiltrating the marrow cavity of numerous bones. It has been found in people within the age group of 30 to 75 yr., the greatest incidence being at the age of 35 yr. It affects the ribs and spine and the upper ends of the long bones, but the flat bones of the skull and pelvis may also be implicated. There are 4 types of myeloma: the plasma cell type, the myelocytoma, the lymphocytoma and the erythrocytoma. At the present time we accept these morphologic variations as evidence of varying degrees of maturation of plasmablasts and consider them to be devoid of any clinical importance. Geschickter and Copeland[838] gave 6 cardinal signs, 2 or more of which they thought were constantly present. These were as follows: "(1) multiple involvement of the skeletal trunk in an adult, (2) pathologic fracture of rib, (3) excretion of Bence Jones bodies, (4) characteristic backache with signs of early paraplegia, (5) an otherwise inexplicable anemia, and (6) lower nephron nephrosis with high nitrogen retention, low blood pressure, and high serum proteins."* Cases have been reported with none of these signs present. Beizer et al.[839] point to other characteristic signs of this disease, as follows: (1) hyperproteinemia, with reversal of the albumin-globulin ratio, (2) hypercalcemia with normal or high serum phosphorus, (3) evidence of autohemagglutination in the counting chamber, on blood films, or detected by the cayenne pepper appearance of blood in the small vessels of the fundus when viewed through the ophthalmoscope while pressure is being placed on the eyeball, and (4) the appearance of an anticomplementary reaction when a complement-fixation test is being carried out. That none of these signs is pathognomonic is obvious.

Mistakes are easy when dealing with a supposed case of multiple myeloma. These cases may be mistaken for metastatic skeletal lesions, hyperparathyroidism, spondylitis, nephritis, and leukopenic leukemia, or vice versa. Quite frequently the suggestion of the existence of this disease comes from a suspicious x-ray picture.

Bone marrow findings

Confirmation may be made by sternal puncture. Rosenthal and Vogel[840] state: "A definite diagnosis of multiple myeloma is possible during life only when made by means of sternal biopsy or the more simple aspiration method of Arinkin."† Beizer et al.[839] studied 10 cases. In 4 cases backache was the principal symptom. In one case, cervical pain was present and in another case arthralgia. Three cases sought relief of an existing anemia. In 7 of the 10 cases, x-ray photographs suggested the diagnosis.

A modified Arinkin method was used by these workers. From 1-1½ ml. were aspirated from the sternal marrow into a paraffin-lined

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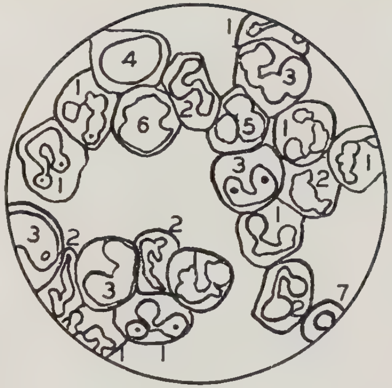
*From Geschickter, C. F., and Copeland, M. M.: Am. J. Cancer **26:** 441, 1936.

†From Rosenthal, N., and Vogel, P.: J. Mt. Sinai Hosp. **4:** 1001, 1938.

Text continued on p. 1400.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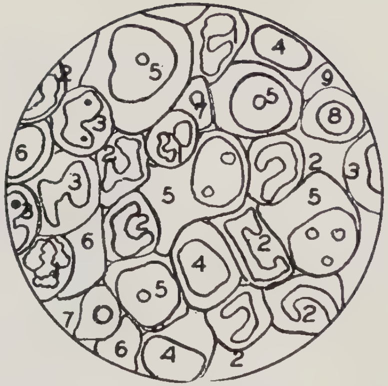
Tuberculosis
Beginning "stab" shift
(Wright-Giemsa stain; ×950)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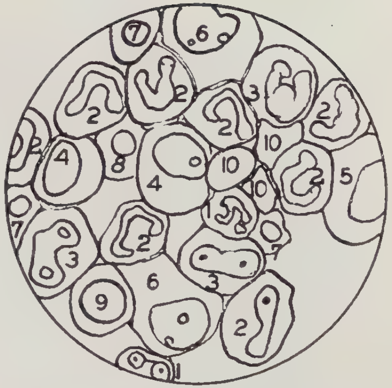
Tuberculosis
"Stab" shift
(Wright-Giemsa stain; ×950)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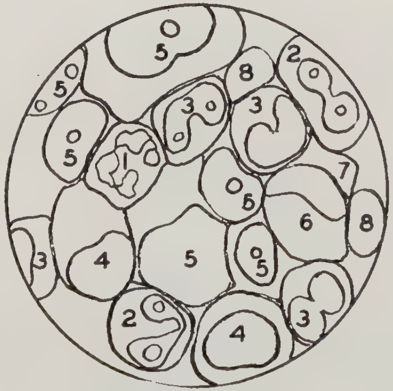
Severe infection
(Wright-Giemsa stain; ×950)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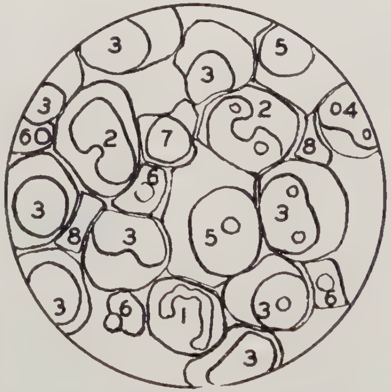
Acute infection
(Wright-Giemsa stain; ×950)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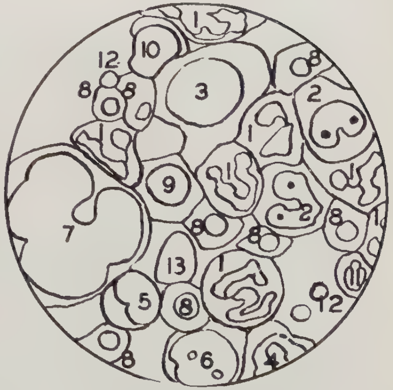
Very severe infection
(Wright-Giemsa stain; ×950)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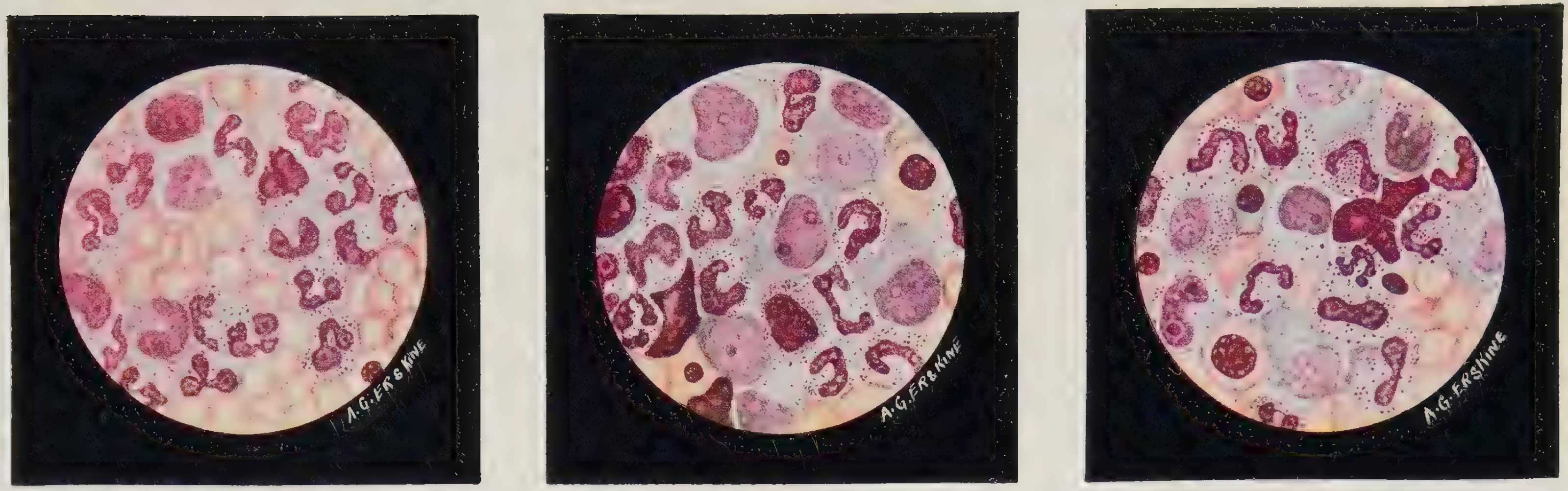
Trypanosomiasis
Guinea pig
(Wright-Giemsa stain; ×950)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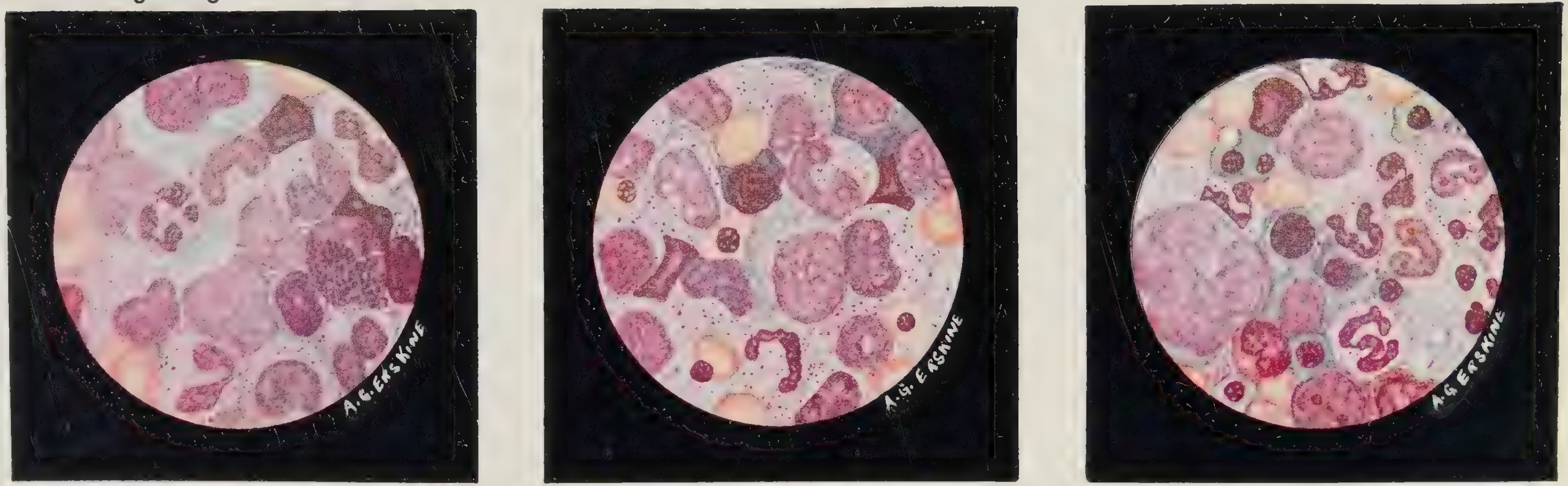
Tuberculosis
Beginning "stab" shift

Tuberculosis
"Stab" shift

Severe infection


A.G. ERSKINE
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A.G. ERSKINE

Acute infection

Very severe infection

Trypanosomiasis
Guinea pig

Fig. 461. Bone marrow touch preparations. For explanation of key see p. 1397.

Fig. 461. Bone marrow touch preparations—cont'd.

Tuberculosis
Beginning "stab" shift

1. Segmented neutrophil
2. Stab cell
3. Neutrophilic juvenile cell
4. Myeloblast
5. Basophil
6. Monocyte (?)
7. Orthochromatic normoblast

Tuberculosis
"Stab" shift

1. Segmented neutrophil
2. Stab cell
3. Neutrophilic juvenile cell
4. Neutrophilic myelocyte
5. Myeloblast
6. Lymphocyte
7. Orthochromatic normoblast
8. Orthochromatic macroblast
9. Polychromatic normocyte

Severe infection

1. Segmented neutrophil
2. Stab cell
3. Neutrophilic juvenile cell
4. Neutrophilic myelocyte
5. Promyelocyte
6. Myeloblast
7. Orthochromatic normoblast
8. Polychromatic normoblast
9. Orthochromatic macroblast
10. Lymphocyte

Acute infection

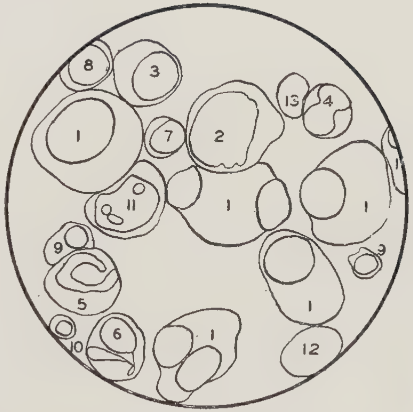
1. Segmented neutrophil
2. Atypical neutrophilic juvenile cells about to segment
3. Neutrophilic juvenile cell
4. Neutrophilic myelocyte
5. Myeloblast
6. Very young eosinophilic myelocyte
7. Lymphocyte
8. Immature nuclear structure

Very severe infection

1. Stab cell
2. Neutrophilic juvenile cell
3. Neutrophilic myelocyte
4. Promyelocyte
5. Myeloblast
6. Orthochromatic normoblast
7. Polychromatic macroblast
8. Lymphocyte

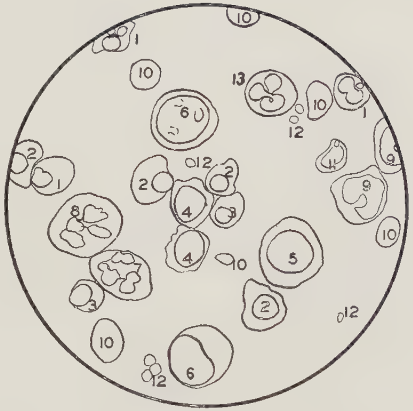
Trypanosomiasis
Guinea pig

1. Segmented neutrophil
2. Neutrophilic juvenile cell
3. Promyelocyte
4. Eosinophil
5. Eosinophilic juvenile cell
6. Myeloblast
7. Megakaryocyte
8. Polychromatic normoblast
9. Polychromatic macroblast
10. Lymphocyte
11. Polychromatic normoblast with beginning karyorrhexis
12. Fat droplets
13. Nucleus of myeloblast



Plasma cell myeloma
(Bone marrow; Wright-Giemsa stain; ×1000)

1. Large plasma cells, two with double nuclei
2. Degenerated promyelocyte
3. Neutrophilic myelocyte
4. Neutrophilic juvenile cell
5. Neutrophilic stab cell
6. Eosinophil
7. Small lymphocyte
8. Polychromatic macroblast
9. Polychromatic normoblast
10. Orthochromatic normoblast
11. Myeloblast
12. Polychromatic megalocyte
13. Polychromatic normocyte

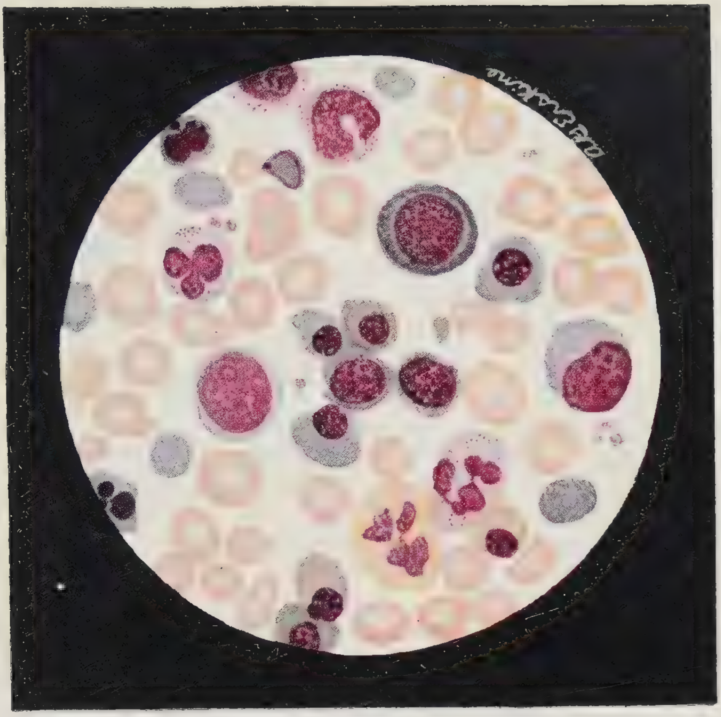


Erythroblastosis
(Blood film; Giemsa stain; ×950)

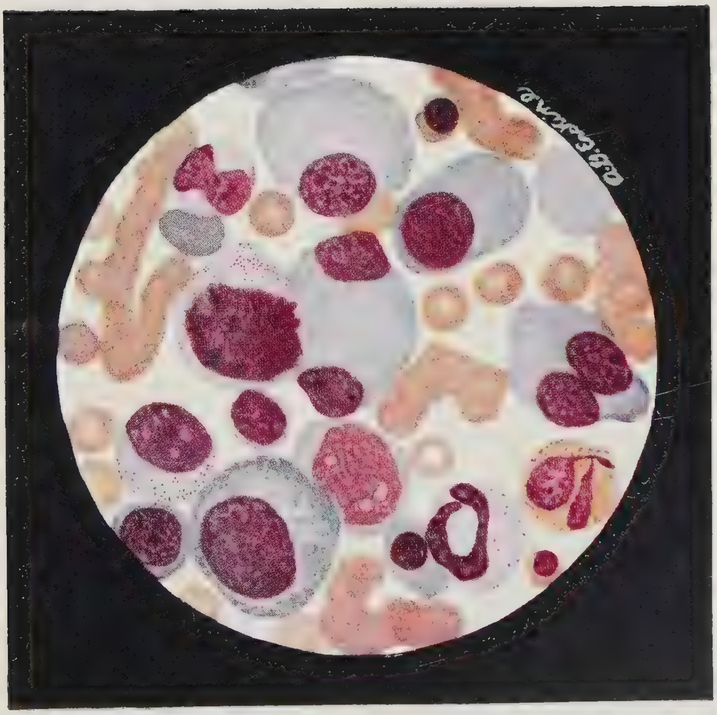
1. Normoblast in karyorrhexis
2. Polychromatic normoblast
3. Orthochromatic normoblast
4. Polychromatic macroblast
5. Polychromatic megaloblast
6. Lymphocyte
7. Segmented neutrophil
8. Eosinophil
9. Neutrophilic juvenile cell
10. Polychromatic erythrocyte
11. Polychromatic erythrocyte with Howell-Jolly and Cabot ring bodies
12. Blood platelets
13. Neutrophilic stab cell

Note anisocytosis with macrocytes

Fig. 462



Erythroblastosis fetalis



Plasma cell myeloma

test tube containing a small amount of heparin. Spreads were stained by Wright followed by Giemsa stain. Diagnosis is confirmed by finding typical myeloma cells. An increase in the typical and atypical plasma cells is also suggestive of multiple myeloma. However, these investigators very properly caution that an absence of these findings in sternal marrow does not rule out the diagnosis.

Myeloma cells show an abundant definitely outlined and deeply basophilic cytoplasm a moderately sized round eccentrically placed nucleus having a fairly coarse chromatin sharply demarcated from the parachromatin, and a very large nucleolus.

Beizer calls attention to the fact that minor variations from this description may exist. The amount of cytoplasm varies from a narrow rim about the nucleus to a large quantity. Multiple small vacuoles may occur. A perinuclear zone may be seen. In one case they found 27.5% plasma cells. These plasma cells had an eccentrically placed nucleus containing coarse clumps of chromatin, and a few cells with somewhat larger nuclei but no nucleoli were seen. They emphasized the fact that the myeloma cell shows a large nucleolus and that in examinations of over 160 bone marrows no such cell was ever found in connection with any other pathologic process. Our own experiences convince us of the accuracy of Beizer's observations as to the specificity of the myeloma cells and also as to the occurrence of many plasma cells in this condition (Fig. 462).

Bayrd and Heck[841] mentioned that a negative diagnosis resulting from sternal aspiration is not always adequate evidence for excluding the possibility of multiple myeloma. Changes in the bone marrow may be irregularly distributed rather than diffuse, and the results on repeated aspirations are not uniform. In one instance in their experience a third aspiration of marrow was necessary to secure a diagnostic specimen.

So far as myeloma cells or atypical plasma cells are concerned, Osgood and Hunter[842] found 45-54% of plasma cells in one case in a 49 yr. old man. Osgood and Hunter considered this to be a true instance of plasma cell leukemia and not multiple myeloma with myeloma cells in the peripheral blood. They held splenomegaly, epistaxis, bleeding from the gums, and the clinical course which this patient pursued to be of the type characteristic of leukemia. As further evidence they cited the lack of Bence Jones proteinuria and of typical defects of the bone in the roentgenograms, bone pain, and bone fragility. Others have felt that this is a distinction of degree only, not of kind. With this opinion Bayrd and Heck concur.

Meyer et al[843] reviewed all the known cases of multiple myeloma in which there were plasma cells in the peripheral blood and all the purported cases of plasma cell leukemia, of which they listed 4. In a careful consideration of these cases they expressed doubt that any were actually cases of true plasma cell leukemia. They rejected these cases largely on the ground that the absence of osseous lesions had not been adequately established. They did report one case which they considered to be a true case of acute plasma cell leukemia although they considered it to "complete the chain in the analogy to lymphoid tumors" such as lymphoma and lymphocytic leukemia.

Diagnosis

The various clinical and laboratory features of multiple myeloma disease are striking, and the occurrence of multiple osteolytic lesions of the bone, Bence Jones proteinuria, and hyperproteinemia is strongly suggestive evidence for the presence of multiple myeloma. However, as pointed out by Bayrd and Heck, every effort should be made to confirm this with pathologic material for the sake of accuracy of prognosis and suggestions as to an outline of treatment. To this end, because of its simplicity and broad diagnostic range, the method of sternal aspiration readily lends itself.

Diagnosis of multiple myeloma can often be made by bone marrow aspiration. In this disease, the normal bone marrow elements are replaced by large numbers of plasma cells. When the percentage of plasma cells is quite high (40-90%), the diagnosis is relatively simple. However, there are many instances in which lower incidences of bone marrow plasmacytosis (up to 20%) may leave some doubt as to the diagnosis. We have had an experience corroborating that of others[844] that an increase in plasma cells may occur in a large heterogeneous group of chronic diseases, such as pulmonary tuberculosis, malignancy (with or without marrow invasion), rheumatoid arthritis, syphilis, sarcoidosis, cirrhosis of the liver, gold intoxication, Banti's syndrome, bacterial endocarditis, periarteritis nodosum, myelofibrosis, chronic leukemias (especially when irradiated), and diseases either members of or simulating collagen disease. A rather interesting feature of many of these cases is that they display disturbances of blood protein relationships, often showing elevations of the globulin fraction. In none of these secondary plasmacytoses have we seen the very abnormal plasma cells found in most cases of multiple myeloma. These myeloma cells, as they are referred to by some observers, are larger than normal plasma cells, ranging from 15-18μ in diameter, and have a slightly pinkish cast to the cytoplasm. However, other cases of myeloma have plasma cells indistinguishable from normal plasma cells. In general, abnormal forms with 2- to 6-lobed nuclei are much more common in myeloma than in the reactive plasmacytoses although we have occasionally found trilobed plasma cells in the latter. Mitoses may be quite common in myeloma and are often abnormal in configuration, as is frequently true in many malignant diseases.

Sections of the bone marrow are helpful in isolated cases of multiple myeloma of the nodular type. In such instances spreads might not show an increase in plasma cells, but the sections may show solid nodules of myeloma cells. In other instances the marrow may be exceedingly fatty, and the spreads give an impression of extreme hypoplasia with very few cells seen. The sections in such cases show the true character of the disease.

The myeloma cells frequently contain various inclusions such as amyloid or paraamyloid, crystalline substances, and ribonucleotides called **Russell bodies.**

Of clinical importance is the so-called single plasmacytoma and the extramedullary plasmacytoma; both lesions, which by varying periods of months to years, precede the development of generalized multiple myeloma.

The serologic and clinicopathologic findings of multiple myeloma are described in the appropriate section.

MACROGLOBULINEMIA OF WALDENSTRÖM

This disease presents a unique mixture of plasmacytic and lymphocytic neoplastic proliferation.[846]

Clinically these patients show hemorrhagic diathesis and enlargement of the liver and spleen and at times of lymph nodes.

Laboratory findings

Hematologic findings: The disease is characterized by an anemia which may vary from moderate to severe. The dysproteinemia is responsible for the hemolytic component, which leads to the appearance of immature red cells in the peripheral blood, and for hemorrhagic manifestations of the disease.

White blood cells: The total white cell count as well as the differential count are usually within normal limits. This point is all the more interesting since the clinical appearance of the patient with enlargement of the liver, spleen, and lymph nodes and with hemorrhagic diathesis readily suggests the diagnosis of leukemia.

Bone marrow: The entire bone marrow is infiltrated by lymphocytes which either are mature or show various transition forms from reticulum cells to plasma cells. The plasma cells are collected in small foci or are distributed diffusely throughout the bone marrow. Frequently there is also an increase in tissue mast cells.

Coagulation work-up: There are a few reports which tend to show defects in various coagulation factors, but it is generally accepted that the bleeding tendency is related to the macroglobulinemia.

Sedimentation rate: The sedimentation rate is markedly increased.

Nonhematologic findings: In addition to the bone marrow findings, the protein changes within the serum[847, 848] are the most diagnostic criteria of macroglobulinemia. The serum contains an increase in total protein which is mainly due to an increase in globulins. Paper electrophoresis frequently shows the appearance of an abnormal globulin which may be found in the alpha, beta, or gamma position or between the beta and gamma bands. It is similar to the peak found in the electrophoretic pattern of multiple myeloma and is therefore labeled M globulin. By methods other than paper electrophoresis, this M glob-

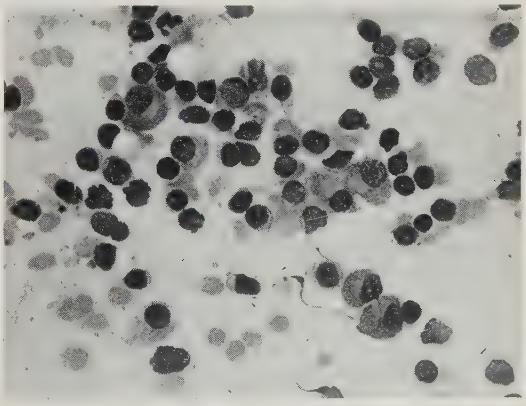


Fig. 463. Bone marrow in macroglobulinemia.

ulin of macroglobulinemia can be separated from the M globulin of multiple myeloma.[849, 850, 850a] Ultracentrifugation characteristically shows an increase in macroglobulins over 5% (normal values, 2-3%). The presence of macroglobulins can also be demonstrated by immune electrophoretic methods. Apart from macroglobulins, the serum may also contain paraproteins and cryoglobulins. The presence of these abnormal proteins leads to a marked increase in the viscosity of the serum which at times is more marked at lower temperatures than at room or body temperature.

The abnormal proteins may lead to false positive serologic tests for syphilis.

Urine: Proteinuria is found.

Laboratory tests for evaluation of serum proteins in macroglobulinemia

Sia test (euglobulin): If 1 drop of serum is added to distilled water, it is precipitated as white, cloudy material, whereas normal serum may produce slight clouding in the distilled water but no precipitate. This test is easy to perform but is not diagnostic of macroglobulinemia since it may be positive in multiple myeloma and other cases of dysproteinemia.

Formol-gel test: Add 1 drop 40% formalin to 0.5 ml. serum. If the test is positive, the serum will gel within a few minutes.

Weltmann band: According to Kappeler et al.[845] the band in macroglobulinemia is shortened, whereas in multiple myeloma it is lengthened.

Pathophysiology

Our present concept is that macroglobulinemia represents a malignant form of lymphoid, plasmacytic, and reticulocytic proliferation. It may occur as a primary malignant form in an acute or more chronic phase. It may also occur secondarily (symptomatically), accompanying such diseases as lymphatic and myelogenous leukemia, lymphomas, various malignant tumors such as carcinomas and sarcomas, and liver diseases.

HODGKIN'S DISEASE

Hodgkin's disease has a fairly high incidence of marrow involvement in studies in which extensive autopsy search has been performed. However in routine bone marrow aspirations, the yield is disappointing. An increase of reticuloendothelial cells with an eosinophilia is suggestive. Rarely, sections of the marrow will reveal the typical Hodgkin's nodule. Out of 46 proved cases of Hodgkin's disease, in only 4 cases were Hodgkin's nodules found, and in all these instances serial sections of the marrow clot were searched vigorously. As with other marrow lesions with a spotty distribution, the success in finding such lesions is dependent in great part on the multiple sites and the search of many sections.

There are scattered reports of Reed-Sternberg cells being found in the bone marrow and peripheral blood. The morphologic identification of these cells is made more difficult by the fact that they resemble young megakaryocytes and that megakaryocytes are frequently increased in Hodgkin's disease. A differentiating feature is that Reed-Sternberg cells are PAS-negative, whereas megakaryocytes readily stain with PAS stain.

LEUKEMIA

Leukemia in almost all forms can be readily diagnosed by study of the peripheral blood. The most common exception to this is the acute stem cell leukemia with a low white blood count. We have found such cases in all age groups from 2 to 84 yr. but have seen it most frequently in the third and fourth decades of life. Organ enlargements, such as splenomegaly, hepatomegaly, and lymphadenopathy, are frequently absent or tend to be minimal. Anemia is usually quite obvious clinically, and the erythrocyte counts are down to 1,500,000 or 2,000,000 red cells/c.mm., with correspondingly low hemoglobin values. Hemorrhagic manifestations are a frequent accompaniment of the disease and reflect striking thrombopenia. Leukocyte values have ranged from 400-2000/c.mm. In all these cases we have been able to find leukemic cells in the peripheral blood, with varying effort required in individual instances. In some of these the leukemic cells have been only a small percentage of the cells in the peripheral blood. The diagnosis of leukemia could be made on peripheral blood studies alone. However, bone marrow aspiration readily corroborated our impression. In all these cases the marrow was exceedingly hypercellular, with an almost complete replacement of normal marrow elements by the leukemic cells. The striking difference between the cellularity of the bone marrow and the peripheral leukopenia in this condition raises a basic question as to what factors determine the delivery of cells from the bone marrow to the circulation. One would certainly expect tremendous leukocytosis in this condition. Perhaps peripheral destruction due to unknown immunohematologic factors plays a role in the leukopenia, anemia, and thrombopenia.

In the **chronic leukemias** there is fairly good correspondence between the peripheral blood changes and the bone marrow picture.

The marrow tends to be hyperplastic, with the increase in cells corresponding with the cell type in the peripheral blood. The degree of shift to younger forms is inclined to be more pronounced in the bone marrow than in the peripheral blood. It is not too unusual to see this disparity in chronic myelocytic leukemia, in which only a few myeloblasts are present in the peripheral blood but the bone marrow may show many such cells. In chronic lymphocytic leukemia approximately 85% of our cases showed typical leukemic hyperplasia. In the other 15%, lymphocytic increases were noted but the marrow was not completely replaced by abnormal lymphocytes. Cases of chronic lymphocytic leukemia, which were effectively treated with x-ray therapy, often showed so few lymphocytes in the bone marrow as to make the diagnosis of leukemia impossible. This was only rarely true in chronic myelocytic leukemia in which even though the marrow became less hyperplastic the monstrosity forms of leukemic development were qualitatively readily identifiable.

Monocytic leukemia, especially in the subleukemic and chronic forms, could usually be diagnosed by the presence of an increase in abnormal monocytes in the peripheral blood. However, our experience with such cases has been that it is not unusual to find only 12-25% of such cells in the peripheral blood. When the increment of increase was in the lower ranges, there were occasions when some doubt was cast on the validity of the diagnosis of monocytic leukemia. In all these cases the bone marrow showed a hyperplastic state due to an overabundance of abnormal monocytes.

The diagnosis of such rare entities as **erythroleukemia (Di Guglielmo's disease)** cannot be made except with the aid of bone marrow aspiration. In addition to hyperplasia of the bone marrow due to an increase in cells of this series, there are qualitative changes in the red cell development of extreme pathologic character. Monstrosities are numerous and consist of abnormal sizes, shapes, and nuclear structures of the red cells. Some cells reach the giant proportions of 35-40μ and have as many as 5-8 nuclei. There is marked asynchronism between cytoplasmic and nuclear development and mitotic figures are often abnormal.

PERNICIOUS ANEMIA

Bone marrow aspiration studies in the anemias are generally not very fruitful from a diagnostic standpoint. Perhaps they are of greatest assistance in the macrocytic anemias, with pernicious anemia the most outstanding example. In this condition the classical bone marrow picture can be seen under the following conditions: (1) a red blood cell count of 3,000,000/c.mm. or less and (2) no administration of erythrocyte maturation factor (liver, folic acid, or vitamin B_{12}). When the red cell count is above 3,000,000/c.mm., the typical bone marrow picture is rarely present, and as the red cell count increases, there is less and less likelihood of finding a typical picture. We have encountered cases displaying the typical neurologic syndrome of pernicious anemia with little or no anemia. Bone marrow aspirations in these cases have been of no help in diagnosis. Insofar as the effect of therapy is concerned, it is well to remember that even modest dosages of erythrocyte maturation factor will quickly alter the bone marrow picture so that within 24-48 hr. after such therapy it may be difficult to make a diagnosis of pernicious anemia. Whenever the diagnosis of pernicious anemia is even suspect, no liver or similar therapy should be administered before the marrow aspiration has been performed. Since such a diagnosis is so important in committing a patient to lifelong therapy, anything that interferes with an accurate diagnosis is to be avoided. Early diagnosis and adequate therapy are the only certain means of avoiding serious neurologic complications. Suboptimal therapy may mask the hematologic picture, whereas these neurologic features stealthily impose themselves on the patient. Once they are firmly established, even the most vigorous treatment may fail. These are but a few of the reasons for accurate diagnosis of pernicious anemia.

The bone marrow in **pernicious anemia in relapse** is markedly hyperplastic. When the marrow puncture is being performed, many cases display tenderness of the sternum. As the marrow material is removed, its hyperplastic character can be seen grossly. Spreads of the bone marrow readily reveal this hyperplasia, which is primarily erythrocytic. The myeloid-erythroid ratio is obviously altered, approaching a 1:1 ratio in most cases and even showing a 1:2 or 1:3 ratio in other instances. Erythropoiesis is of the primitive type, simulating that of the embryo in the prehepatic stage. There is a marked shift to younger forms as evidenced by the appearance of megaloblasts. Not only is there seen a profusion of megaloblasts, but there is also noted a marked increase in other young forms, such as basophilic normoblasts and polychromatophilic normoblasts. Pathologic erythropoiesis reaches its greatest proportions in the bone marrow in pernicious anemia, being approached only in the rare cases of Di Guglielmo's disease or other leukemic disease. These pathologic changes affect the cytoplasm as well as the nuclei of the red cells and consist of the following changes.

Abnormal cell size: The megaloblast itself begins as a giant form, usually measuring 20-24μ in diameter. There is little wonder, then, that the offspring of this cell are large. This developmental defect accounts for the macrocytosis.

Asynchronism of development: In normal cell development, cell size, cytoplasmic staining, and nuclear size keep pace in an orderly manner. In pernicious anemia, this synchronism disappears. Large cells with small nuclei are common. Many cells may display an extremely basophilic cytoplasm and have the nuclei of fully developed normoblasts.

Karyorrhexis of nuclei: Fragmentation of nuclei at all stages of development is readily apparent. This may vary from simple binucleated cells to erythrocytes containing one large nucleus surrounded by single or multiple Howell-Jolly bodies. Other red cells may display Howell-Jolly bodies only.

Karyolysis of nuclei: Whereas normal nucleated red cells show karyolysis of the nuclei by a diminution in size, the erythrocytes of pernicious anemia frequently display this phenomenon within the nucleus. As a result, the stained nucleus takes on a soil erosion appearance, showing alternating areas of well-stained chromatin alongside areas which do not stain at all or stain a reddish hue. Not only can this phenomenon be seen in sizeable nuclei, but it is also often visible in Howell-Jolly bodies.

Mitotic activity: This is exceedingly great and is abnormal. The abnormalities take on many bizarre features. It is not unusual to see 1 or 2 chromosomes off by themselves in one part of a cell away from the the major mitotic figure. When the mitotic figure shows separation of the chromatin, 1 pole may show considerably more material than the other. Abnormal condensations of chromatin are often noted, and lysis of the chromatin in mitotic figures (just as in the intact nuclei) is observable.

There is a feature of pernicious anemia bone marrow that is mentioned fairly frequently but is rarely stressed, even in hematology texts, and this relates to the **abnormal leukocyte development** in this disease. This pathologic myelopoiesis is as regularly occurring in pernicious anemia as are the red cell changes, and it disappears with specific therapy just as promptly as the abnormal erythropoiesis. The most striking abnormality is the finding of giant white cells, particularly notable at the promyelocyte, myelocyte, and juvenile stages, reaching a size of 20-22μ in diameter. In many of these cells there is often an inordinately great amount of nuclear material, giving the impression that the cell has all that it can do to contain the nucleus. When such cells go on to full maturity, they typify the pernicious anemia neutrophil with its increased size and multilobulated nucleus. Mitoses in the myelocytic series are increased and occasionally are abnormal.

The only other macrocytic anemias that we have encountered which are indistinguishable from pernicious anemia have been observed in tropical and nontropical sprue and in a rare case of *Diphyllobothrium latum* infestation. In all other instances, the differential diagnosis has been relatively simple. Advanced liver disease, such as cirrhosis, is occasionally associated with a macrocytic anemia. In none of the cases we have studied have we seen the degree of pathologic erythropoiesis and myelopoiesis observed in pernicious anemia in relapse. Macrocytosis may be striking in such cases but the other pathologic features are lacking. This has been true in cases of carcinoma (especially of the stomach), pernicious-like anemia of pregnancy, and some refractory anemias.

MICROCYTIC AND NORMOCYTIC ANEMIAS

Insofar as anemias other than the macrocytic anemias are concerned, bone marrow aspiration is rarely diagnostic. The anemias of chronic hemorrhage and of hemolytic disease often show nonspecific normoblastic hyperplasia of varying degree. This can be demonstrated in both spreads and sections of marrow. Aspiration of bone marow in these cases is at times helpful in eliminating other diagnoses, but the positive aids in these anemias come chiefly from the history, physical examination, and peripheral blood studies.

There is a group of anemias in which **sections** of bone marrow material have given us more information than do the **spreads** of the same material. Included are the anemias associated with malnutrition, terminal nephritis, and advanced liver disease. In all these instances the sections of marrow show a marked increase in fatty marrow which replaces normal marrow. The marrow hypoplasia may become so marked that only isolated islands of bloodforming tissue can be seen interspersed between large fat globules. Both sections and smears can be stained for the presence of free iron. This procedure, as mentioned in the hematology section, can be extremely helpful in classifying anemias.

Aplastic anemia and myelofibrosis

In these states the bone marrow may become exceedingly hypocellular or almost completely replaced by fibrous connective tissue. Some comment has already been made concerning the relative merits of spreads and sections in these conditions, with the section technic holding the upper hand. If, however, successive dry taps are encountered, surgical trephine should be performed before such a diagnosis is made.

In addition to these idiopathic states, we have encountered variable degrees of bone marrow fibrosis in other conditions. Chronic blood dyscrasias such as leukemia and polycythemia which have received significant amounts of radiation therapy may show a considerable degree of fibrosis. The same is true of Hodgkin's disease treated with radiation or nitrogen mustards. Some metastatic carcinomas show extensive fibrous replacement of bone marrow. We have observed this to be particularly true in cases in which we were able to demonstrate capillaries plugged with tumor cells. In most of our cases of metastatic carcinoma this was not true; normal marrow units were seen alongside large aggregates of tumor cells. A few cases of sarcoidosis and a rare case of widespread tuberculosis showed marked replacement of normal marrow.

POLYCYTHEMIA

In polycythemia vera, there is hyperplasia of all marrow elements which can be quantitatively appraised better in sections than in spreads. Block et al.[851] have used the appearance of sections as a guide for therapy in polycythemia vera. If the marrow sections show marked hyperplasia, they recommend further active radiation therapy, regardless of the peripheral blood values. We have noted an interesting and undescribed phenomenon in polycythemia vera related to the megakaryocytes. These cells show an unusual degree of cytolysis with all the cytoplasm of almost every megakaryocyte showing platelet formation and leaving bare nuclei surrounded by platelets. This phenomenon might account for the thrombocytosis commonly associated with polycythemia vera and has not been observed in the secondary polycythemias.

MALIGNANT LYMPHOMAS

Malignant lymphomas at times may be diagnosed by bone marrow aspiration. For this diagnosis we still prefer biopsy of lymph nodes over bone marrow aspiration because the former will yield a higher return than the latter. Table 277 requires a word of explanation. The 100% diagnosis under sections as compared with only 9.4% positives in spreads represents comparative figures between the 2 technics. We have not found that 100% of lymphosarcomas can be diagnosed by marrow aspiration but that when the diagnosis is made this way, the sections of marrow will show it 10 times as frequently as the spreads. This disparity between the 2 technics is readily comprehensible when one observes numerous sections of such cases.

Lymphosarcoma has a tendency to form **nodules** in bone marrow rather than to be diffusely spread throughout the marrow. The usual picture is that of a central nodule, consisting solely of lymphosarcoma cells, within a marrow unit, the periphery of which consists of normal marrow elements. Not infrequently this peripheral zone shows a marked eosinophilia. Alongside such affected marrow units may be seen perfectly normal units and these generally outnumber the involved areas. In the preparation of spreads from such material, if one does not happen to catch a nodule of lymphosarcoma cells, the diagnosis will be missed. We have the impression that these lymphoid nodules are held together rather compactly and do not spread well. In the spreads in many of these cases, one may encounter a moderate increase in abnormal lymphocytes but only of sufficient degree to arouse suspicion of the diagnosis. A generalization that appears reasonable from our observations is that when there is an increase in abnormal lymphocytes in the peripheral blood, the bone marrow is likely to show a diffuse spread of these cells. This is the pattern of chronic and acute lymphocytic leukemia and of leukosarcoma. As a corollary to this observation, when we see the nodular type of lesion in the bone marrow, abnormal lymphocytes are then rarely seen in the peripheral blood. At times we have observed one type transform into the other, most frequently the nodular type changing into the diffuse type of disease.

A word of caution is in order concerning lymphocytic nodules in bone marrow. Isolated nodules may be seen in normal bone marrows and in our experience are fairly regularly encountered in elderly persons, particularly those with malnutrition. The finding of an isolated lymphocytic nodule, therefore, does not make a diagnosis of lymphosarcoma. We insist on the finding of goodly numbers of such nodules before making this diagnosis. In well-prepared thin sections, it is often possible to identify individual lymphocytes which are abnormal. An increased incidence of mitoses adds further weight to the diagnosis.

We have found bone marrow aspirations helpful in 2 situations in which lymphosarcoma is suspect. By far the largest number has been those patients who have mediastinal or abdominal involvement and no readily accessible peripheral lymphadenopathy. Marrow aspiration in these cases is a much simpler approach to diagnosis than thoracotomy or abdominal exploration. The other situation in which we have found marrow aspiration useful is in determining the extent of involvement of the disease. We have observed patients with lymphosarcoma of the skin, with no significant organ enlargements or lymphadenopathy, who have shown extensive bone marrow involvement. In these cases we have recommended more vigorous therapy than would have been used for the skin lesions themselves. Prognostically, patients with marrow involvement have had a poorer outlook than those without such spread of the disease.

INFECTIONS

The usual response of the bone marrow in acute infection is moderate hyperplasia of the myelocytic elements with a left shift in this series paralleling the shift seen in the peripheral blood. At times this shift may reach the proportions seen in chronic myelocytic leukemia. There are several helpful differential points in the bone marrows of these 2 conditions, as listed in Table 279.

Table 279

	Infection	*Myelocytic leukemia*
Toxic changes in leukocytes	Present	Absent
Monstrosity forms	Absent	Present
Increased basophils	Absent	Often present
Hiatus phenomenon	Absent	Often present
Myeloblasts	Few	Usually plentiful

In the field of chronic infections with specific granuloma lesions, bone marrow studies may make the diagnosis. Brucellosis, tuberculosis, and histoplasmosis are examples. The success in finding these lesions is dependent on the multiplicity of punctures and the vigor with which the search for them is conducted. Obviously, sections of the bone marrow are superior to spreads. Granuloma lesions such as sarcoid belong in the same category.

Spreads may be helpful in finding the organisms of leishmaniasis, histoplasmosis, and toxoplasmosis. Although articles have appeared suggesting bone marrow studies for malarial infestations, we have not found this procedure necessary. After an extensive experience with malaria in the Armed Forces, Agress' recommendation in this search for malaria is thorough knowledge of the thick drop technic. He was not able to find a single instance of malaria in which he could demonstrate the parasites in bone marrow material and not find them as readily in thick drop preparations.

Culture of bone marrow for isolation of certain organisms has been successful in isolated instances such as brucellosis and tuberculosis.

CONTRAINDICATIONS TO MARROW ASPIRATION

Bone marrow aspiration is not indicated until an adequately complete history, physical examination, and peripheral blood studies have been performed and indicate the desirability of the procedure. In adition to the discomfort to the patient, the procedure carries some danger, a few deaths having been reported as a result of bone marrow aspirations. Perhaps the only medical contraindication to bone marrow aspiration is hemophilia in which uncontrollable bleeding may follow the puncture.

TECHNIC OF BONE MARROW INVESTIGATIONS

The first instrument for the examination of bone marrow was made by Wolff[852] in 1903. This examination was performed by trephination of the diaphyses of the tibias of small animals. Human bone marrow of a living subject was first examined by Ghedini[853] in 1908. This method, following the method of Seyfarth, was used by Schilling and Gradwohl for some time, but has been superseded by the relatively simple process of direct sternal puncture instead of trephining as we previously performed this technic. The difficulties incident to an operation such as trephination were removed by Arinkin[854] in 1929. He used a large needle and trocar and punctured the body of the sternum under aseptic conditions, using a local anesthetic.

Gradwohl[855] reported a new instrumentarium for performing sternal puncture. It consists of a 10 ml. Luer-Lok syringe, a cannula and connector with a rubber tubing joint, a bone marrow puncturing instrument with a guard which can be adjusted to fit the depth of the sternal puncture, and an obturator for the latter.

The skin over the sternomanubrial junction is surgically prepared, the usual sterile drapes are applied, and local anesthesia with procaine hydrochloride solution is injected intradermally and subcutaneously and into the periosteum. The instrument for puncture has a handle which permits a firm grasp and allows one to bore through the external lamina of the sternum and enter rapidly the marrow cavity of this bone. The instrument is thrust through the skin at the sternomanubrial junction at right angles to the surface of the sternum. The guard on the shaft of the needle is so adjusted that the instrument cannot possibly be pushed through the sternum into the mediastinal space. After the instrument has well penetrated the sternum, the direction is changed with the point directed upward. At this time, when the bone marrow has been entered, the obturator is removed, the connector and syringe are attached above the handle of the boring instrument, and suction is applied. One obtains in this way a very definite specimen of the bone marrow. It goes without saying, of course, that the entire instrumentarium must be adequately sterilized before use. One may place a small amount of dilute sterile sodium citrate solution in the barrel of the syringe, which slightly dilutes the bone marrow material.

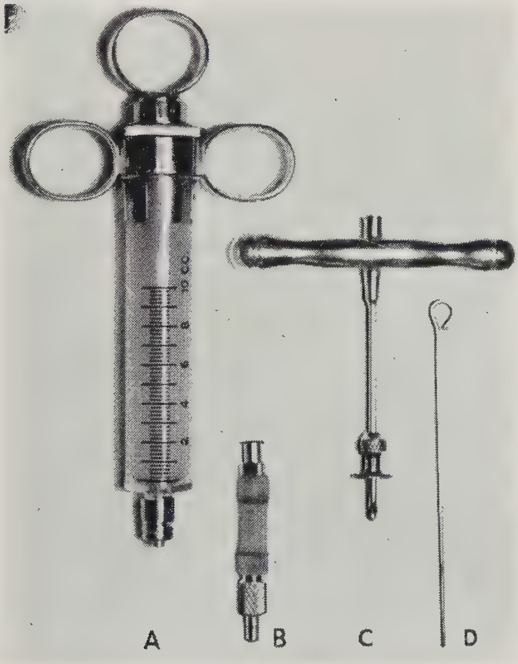


Fig. 464. Gradwohl-Aloe sternal marrow puncture apparatus: **A,** syringe; **B,** cannula and connecting tubing; **C,** bone marrow puncture instrument; **D,** obturator.

The large caliber of the instrument permits one to obtain sufficient bone marrow easily. Once the marrow is obtained, the instrument is rapidly removed, and the opening is closed with a surgical dressing. Repeated punctures on the same patient can be made with this instrument without any difficulty.

It is not necessary to remove a large quantity of bone marrow. Only a small amount is obtained, usually about 0.2-0.3 ml.

Fig. 464 shows the entire setup for the procedure.

Biopsies of sternum—Turkel technic

The Turkel trephine biopsy method[856-863] eliminates the 2 common objections to marrow biopsies: (1) that biopsy is a surgical procedure and (2) that biopsy leaves a disfiguring scar.

The advantages of this method are that it is easily and rapidly performed at the bedside or in the office and does not leave a disfiguring scar. It controls the depth and force of penetration; in other words, it prevents penetration of posterior lamella and mediastinum. There is no dilution of bone marrow with circulating blood. There is preservation of bone marrow topography. Biopsy by this method shows cells adherent to the marrow wall which are not seen by aspiration. Further the biopsy shows the true proportion of cells in the bone marrow or the myeloid-erythroblast ratio. It checks results of treatments quantitatively. It possesses the advantage that aspiration and infusion may be performed through the same aperture.

Procedure:

1. Cleanse the skin over the desired site, usually opposite the third rib, with surgical antiseptic. Infiltrate skin, subcutaneous tissue, and periosteum with local anesthetic.
2. Insert outer needle with stylet in place (Fig. 466) with the tip of the needle in the direction of the face of the patient at an angle of about 45° through the skin, subcutaneous tissue, and periosteum until the tip just engages the anterior lamella of the sternum.
3. Leave the outer needle in place and remove the stylet.

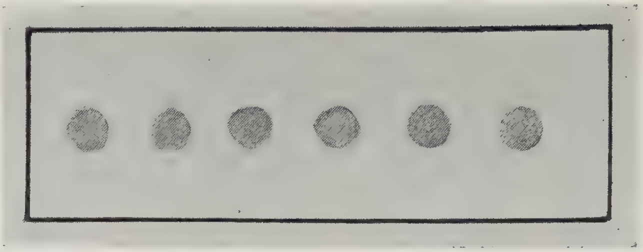


Fig. 465. Bone marrow touch preparation.

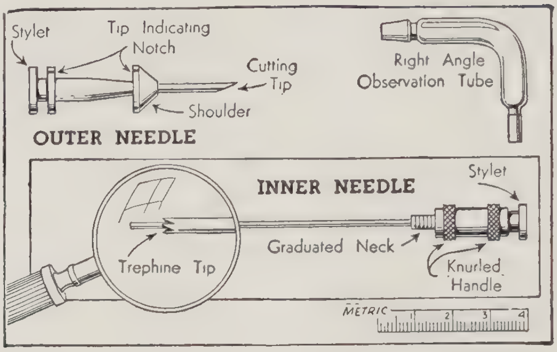


Fig. 466. Turkel needle for aspiration or biopsy of sternum.

4. Insert the trephine needle with stylet into the outer needle.
5. Remove style of inner trephine needle.

While holding the outer needle with the fingers of the left hand, turn the head of the trephine needle with the fingers of the right hand, with a slight clockwise-counterclockwise motion, at the same time exerting gentle inward pressure. A sudden release of resistance signifies entrance of the trephine tip into the sternal cavity. **The clockwise-counterclockwise motion, with slight pressure, should continue until the neck of the inner needle is completely within the head of the outer needle.** Rotate the head of the inner needle in order to detach the plug from the surrounding marrow.

For aspiration:

1. While holding the knurled handle of the inner needle, push the outer needle into the sternum about 6 mm. until the neck of the inner needle becomes completely visible.
2. Remove the inner needle.
3. Attach the syringe and aspirate about 1 ml. blood-marrow mixture.
4. Remove syringe and express mixture immediately into oxalated tube.

For biopsy:

1. Remove the inner needle and with its stylet force the marrow plug into a bottle containing a fixative or use it as imprint or spread.
2. Insert stylet into outer needle, remove outer needle, and cover the point of insertion with antiseptic dressing.

Biopsy of tibia

In infants and younger children it is safer to utilize the tibia instead of the sternum in obtaining bone marrow specimens. Except for the location, the technic is the same as that used in sternal puncture.

Use of iliac crest in bone marrow punctures*

Bone marrow can be obtained easily and safely from the iliac crest and may at times provide information not obtainable from sternal aspiration.

Procedure: The site for the iliac crest puncture is chosen on the crest over an area within 3 cm. posterior to the anterior superior iliac spine on either side of the body. According to the thickness of the tegument a needle 1 to 2½ in. long, 16 gauge, and furnished with a stylet is used for puncture; a 20 ml. tightly fitted dry syringe is used for aspiration.

The determined site is prepared with iodine and alcohol and infiltrated with 2% Novocain solution until the periosteum is reached; the periosteal space is then injected very slowly; usually no more than 2 ml. Novocain solution are required. The patient lies in the supine position; in very pronounced abdominal distention (ascites, enlarged viscera) the patient is tilted on his side. The puncture is performed with the needle held at a 45° angle to the long axis of the body and with pressure applied by the palm of the hand. The aspirating needle is forced into the bone with steady pressure and slight rotation. A distinct give sensation is usually felt when the needle enters the medullary cavity. As the needle is firmly embedded in the medullary cavity, the stylet is withdrawn, and the syringe is attached to the needle. In order to avoid admixture of blood the plunger is slowly withdrawn until the first drop of marrow fluid is observed in the capillary end of the syringe. Bone marrow aspiration is accompanied by a slight sensation of pain (suction pain).

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*Michael A. Rubinstein, Montefiore Hospital, New York, N. Y. Personal communication based upon facts by Dr. Rubinstein in his Scientific Exhibit, A. M. A. convention, Atlantic City, N. J., June, 1947.

The material removed may be used in the usual way for enumeration of total nucleated cell count, for preparation of smears, and for section study.

The technical advantages of iliac crest versus sternal puncture are as follow:

1. No injury can be sustained by any underlying organ.
2. Usually the iliac puncture is a less painful procedure and the patient is less apprehensive than when subjected to the puncture in the cardiac area.
3. Repeated aspirations of the iliac crest can be easily performed at frequent intervals on both sides; iliac aspiration is very suitable for serial bone marrow studies. This may be important especially in experimental pharmacologic physiologic research of hematologic tissue and bone marrow tumors.

It is suggested that the marrow cavities of the iliac bone can be used as a readily available route of administering fluids into the general circulation when no intravenous route is accessible since they allow for greater safety than the sternal technic.

Disadvantage of the iliac aspiration is the occasional difficulty of puncturing the bone, which at times may prove to be exceedingly firm in this region.

Bone marrow studies were performed simultaneously in iliac crest and sternal aspiration in 216 different cases showing normal and pathologic findings.

Normal: The normal values of the iliac bone marrow were found to be approximately in the same range as those determined for sternal aspiration. Iliac crest counts in hematologically active bone marrow in all age groups of normal individuals studied (age range 16-78 yr.) gave the following results. The total nucleated cell count of the iliac bone marrow, as that of sternal aspiration, tends to decrease with age from the average of 110,000/c.mm. in persons under 20 yr. of age to 50,000/c.mm. in those over 60 yr. of age. The differential count of the normal iliac marrow ranged as follows: myeloblasts, 0.5-2%; promyelocytes, 1-5%; myelocytes, neutrophilic, 11-23%; myelocytes, eosinophilic, 1-3%; myelocytes, basophilic, 0.5-1%; nonsegmented neutrophils, 8-29%; segmented neutrophils, 5-15%; eosinophils, 1-3%; basophils, 0.5-2%; lymphocytes, 2-10%; plasma cells, 0.5-3%; reticulum cells, 0.5-2%; hematogones, 0-3%; proerythroblasts, 0.5-1%; erythroblasts, 2-8%; normoblasts, 10-21%. The number of megakaryocytes ranged between 22 and 88 in 1 c.mm.

Examination of bone marrow preparations

Touch preparations are made on the surfaces of clean fat-free glass slides. A few spreads also are made.

The combination of Wright and Giemsa stain is the best staining method. The specimens are dried in the air and stained with Wright stain 1 min., followed by differentiation in neutral distilled water for 5 min., and then washed. The specimens are then stained for 30 min. with a dilute Giemsa stain made by adding a drop of stock Giemsa stain to each ml. neutral distilled water.

After the staining process is completed, the slide is washed with neutral distilled water, dried in the air, and examined. This combination stain gives excellent results.

In connection with the technic of bone marrow investigations, which was just dedescribed, the method used by Isaacs[864] to count the cells of the bone marrow is of inestimable benefit in the determination of the type of anemia. The description is as follows:

Wet the inside of the tube of a Sahli hemoglobin pipet or the stem of a red or white blood cell counting pipet with serum. The pipet stem should have markings enabling one to measure 4 vol. A serum of the same blood group as the patient is desirable if the red blood cell count is a factor; otherwise any blood serum may be used. The serum is drained out, and bone marrow is sucked up 1 unit of space into the pipet. By working the tip back and forth among the trabeculae of bone, enough marrow may be obtained without air bubbles. Serum is then drawn up so that the total bulk is 4 vol. This is then discharged into a watch glass, using care to wash out all of the marrow. Eight more vol. serum (measured by the markings on the pipet) are then added, and the suspension is stirred thoroughly with a blunt glass rod. This suspension is then drawn into a red blood cell counting pipet to the 0.5 mark and is diluted with Hayem solution or with 1% acetic acid if a count of leukocytes only is desired, the rest of the steps being exactly as in counting red blood cells using a hemacytometer slide.

The high dry objective is used. The total number of cells in 4 or 8 sq.mm. are counted, the adult red blood cells being enumerated in 1 group (if acetic acid has not been used) so that when their number is subtracted from the total, the number of nucleated cells of all types is obtained. The calculations are as follows.

The number of cells counted in 1 sq.mm., obtained by dividing the total number of cells counted by the number of sq.mm. spaces used, is multiplied by 24,000, i.e.:

$$\frac{10 \times 12 \times 200}{\text{No. sq.mm.}}$$

The differential count is made from cover glass films, using a thick emulsion of bone marrow in serum. The fresher the serum, the better are the results. If the serum is of the same blood group, the red blood cells are not agglutinated but are evenly distributed. Human serum may be used for bone marrow of animals. Use Wright or Giemsa stain. When Wright stain is used, allow the stain to act for 1 min. and then dilute with as much distilled water as the cover glass will hold, mixing very thoroughly. Allow this to stand for 10 min., then wash with distilled water, dry, and mount in xylol-balsam.

The most representative bone marrow is obtained from the midsternum, as this gives both red and white cells in the course of their development. This technic may be used with marrow from any bone or for cells of other organs.

With careful technic, the variation in counting cells from the same region in the normal marrow using several pipets averages about 5%, an amount but slightly greater than variations from drop to drop in the same pipet. In pathologic marrows, when there is a grossly uneven distribution of the cells, the variation is considerably greater, amounting to as much as 24% between a cellular and relatively noncellular region and to 85% in marrows with regions of aplasia.

With this method Isaacs maintains that there are from 150,000-300,000 cells/c.mm. in normal sternal bone marrow. He states that the primitive blast stage of the cells of the polymorphonuclear leukocyte series resembles that of the lymphocyte and erythrocyte series of cells in general morphology and may or may not be identical. The next stage in the development of the red blood cell he terms the megaloblast and believes that this is a normal stage in the production of the erythrocyte rather than a pathologic or fetal cell, as many other authorities believe. He calls the next stage in development the basophilic normoblast or macronormoblast. Isaacs calls attention to the variation in the number of cells in the bone marrow dependent upon changes in physiology. In other words, a normal individual may tax his granulopoietic tissue at one time more than another in trying to remain normal. His view of the normal composition of the cells of bone marrow is shown in Table 280.

Table 280

Cell type	*Per cent all nucleated cells*
Undifferentiated lymphoid cells (primitive blasts)	23.1 ± 8.0
Megaloblasts	3.0 ± 1.0
Basophilic normoblasts	7.2 ± 2.5
Eosinophilic normoblasts	12.0 ± 7.0
Polymorphonuclear neutrophils (adult)	7.5 ± 5.5
Polymorphonuclear neutrophils (young)	13.0 ± 6.0
Metamyelocytes	20.8 ± 3.0
Myelocytes	2.8 ± 1.8
Myeloblasts	1.8 ± 0.8
Eosinophils (immature and mature)	5.5 ± 3.0
Lymphocytes	1.0 ± 0.5
Endothelial cells (phagocytes)	0.9 ± 0.5
Hemohistioblasts and hemohistiocytes	2.3 ± 1.3
Megakaryocytes	0.5 ± 0.1
Basophils	Present
Monocytes	Present

He calls attention to the fact that in aplastic anemia the total number of cells per ml. is greatly reduced, and the fat content is increased. So-called idiopathic aplastic anemia differs from aplastic anemia following intensive roentgen ray therapy in that in the latter condition the number of primitive blasts is reduced, but the cells which are present tend to ripen into eosinophilic normoblasts and later into mature erythrocytes. In the hypoplastic anemia of nephritis the inhibition is just after the primitive blast stage so that cells in this stage accumulate in normal or greater than normal numbers, whereas but few ripen to the megaloblast stage.

In pernicious anemia he found that the point of inhibition appears at the megaloblast stage. Inhibition appeared at the normoblast stage in leukemia and infection.

The next stages are eosinophilic normoblast, reticulocyte, polychromatic red blood cell, and the mature erythrocyte.

Agress modification for preparing spreads and sections simultaneously

1. Perform marrow aspiration in the standard manner, using the site of the operator's choice or as indicated in individual problems.
2. Attach a **dry**, sterile, tight-fitting 5-10 ml. syringe to the aspirating needle.
3. Make a **strong, sudden** pull with the plunger, withdrawing only about 0.5-1 ml. of marrow. This first show of marrow generally is the most cellular and has the least dilution with blood.
4. Quickly transfer the entire contents of the syringe to a clean glass slide.
5. Touch this pool of marrow by the edges of successive slides and then spread over individual slides to make the spreads.
6. Generally 8-12 spreads should be made of each preparation.
7. Stain spreads as outlined on p. 1409.

When the pool of marrow has clotted, pick up the clot with the stylus of the aspirating needle, and transfer to a fixative. Thereafter treat like a routine surgical biopsy. Our preference in fixatives is Zenker's acetic acid (10%), which permits the use of special stains if desirable. The routine staining is hematoxylin and eosin, with particular care exercised in differentiation.

Using this method, Agress has found that 92% of spreads and 95% of sections are satisfactory.

Anticoagulants are **not used** at any time in the routine preparation of bone marrow spreads or sections. However, lupus erythematosus (L.E.) cells will only rarely be found when this technic is used. If L.E. cell preparations are desired, the method differs.

For L.E. cells:

1. Leave the aspirating needle in situ.
2. Aspirate 1-3 ml. marrow into a syringe containing a few drops of heparin or other anticoagulant.
3. Allow this material to incubate at 37° C. for 2 hr.
4. Centrifuge at 1000 r.p.m. for 5 min.
5. Make spreads of the buffy coat, and stain (p. 1140) in the routine manner.

For cultures of bone marrow: If cultures of bone marrow are desirable, these too are collected after the routine method has been performed, leaving the aspirating needle in place. A clean sterile syringe containing sterile anticoagulant is reapplied, and an effort is made to obtain as much as 5 ml. marrow material. This is then transferred to appropriate media.

TISSUE CULTURE OF HUMAN BONE MARROW

Osgood has carried out some very interesting research work on cultivation of bone marrow. He has presented this work in various communications.[865-867] This method was used by him for the appraisal of therapeutic agents. It permitted determination of their effect on living human cells, of the toxic dose, and of the minimum therapeutic dose as well as the accurate control of concentration. He showed by his culture method a number of points about the length of life of neutrophils, eosinophils, and basophils of normal blood by comparing blood and sternal bone marrow from healthy persons. The cultures were made in 30 ml. vaccine vials in a medium consisting of about 35% of human cord serum and 65% of the balanced salt solution of the Geys.[868] This medium was previously determined by the Geys to be satifactory for the culture of human tumors. Osgood found it possible to maintain cultures of human marrow in as nearly their natural state as any medium so far investigated.

Method: Assemble the apparatus from 5 two-liter aspiratory bottles,* Pyrex standard wall glass tubing, and pressure tubing. Test its operation. Determine the volume of fluid in bottle 1 at the level of the inlet orifice of 4. Connections must be gas-tight under a pressure of at least 60 in. water. Determine the number of drops/ml. from tips 21 and 11. With water instead of medium flowing through the apparatus at the desired rate for use with the marrow, adjust the temperature of the water bath so that the temperature as determined on the fluid within 1 after a period of time for equilibrium to be reached is 37.5° C., the optimum temperature for the experiment. The temperature in the flask is usually about 1° lower than in the bath, but it should be determined for each experiment. Now disassemble the apparatus at convenient joints. Sterilize bottles 1, 5, and 9 with their connections as far as 23 in the autoclave, wrapping the free ends in paper or gauze. The gas system, including bottles 13 and 19 and their connections, need not be sterile. Reassemble the apparatus using aseptic technic, flaming the ends of glass tubes and pouring 70% alcohol on the rubber tubing before making each connection. Introduce the marrow, obtained as described by Osgood,[869] into bottle 1 through vaccine cap 2.

Introduce medium[869] with syringe and needle through rubber tube 7, previously sterilized with alcohol, into bottle 5. Close screw clamp 8. Force air in through 6 until the medium is all transferred to reservoir bottle. Use compressed air or a blood pressure manometer bulb. Disconnect bulb from 6 and adjust the flow of the medium from reservoir 9 by screw clamp 10, counting the number of drops/min. from tip 11, previously calibrated so that the ml./hr. or 24 hr. can be calculated. Any desired gas mixture can be made up in a spirometer and introduced into bottle 13 by closing screw clamps 14 and 15 and filling bottle 13 completely full with 5 to 10% sulfuric acid.* Attach the outlet of the spirometer to inlet 16 and allow the gas to flow into bottle 13 by opening screw clamps 17 and 18, collecting the acid as it flows out in reservoir bottle 19, which is disconnected for this purpose.

When 13 is almost empty, close 18 and 17, place 19 back on its shelf, and attach to outlet 20. Open 14 and adjust the rate of flow of acid by the number of drops/min. of calibrated tip 21 to give the desired rate of gas flow. Open 15. Filter the gas leaving bottle 13 through sterile cotton 23, pass through trap 24; in passing down tube 3, it equilibrates with the medium flowing through the same tube as it enters culture bottle 1.

Medium[869] and gas enter through fine tip 3. The medium, being slightly cooler than that in the flask, flows across the bottom and then gradually rises to the surface. Pressure of inflowing medium and gas forces the used medium and gas out through tube 4 into reservoir 5, from which the gas escapes through cotton-filled bulb 6. It may be collected if desired.

If desired, tilt culture bottle 1 at any time so that the inlet of 4 is above the level of the liquid. Thoroughly mix by shaking, and aspirate marrow culture through 2 for counts and examination.[869] Calculate total numbers of any cell type present by multiplying the number/c.mm. by vol. culture in c.mm. No semipermeable membrane is necessary because the cells settle out and only the surface layer of fluid flows out through tube 4.

With this method, a rapid, controlled rate of flow of the medium past the cells and a rapid, controlled rate of gas supply is possible. Medium from bottles 1, 9, or 5 may be aspirated with a sterile syringe and needle through the pressure tubing at the outlet at any time for chemical, bacteriologic, or serologic examination. Medium may be used several times by pumping it as described from bottle 5 to bottle 9. Gas may be collected at 6 for analysis.

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*Any size container may be substituted for these without altering the principle of the method.

*This prevents growth of molds and bacteria and dissolves less carbon dioxide than water.

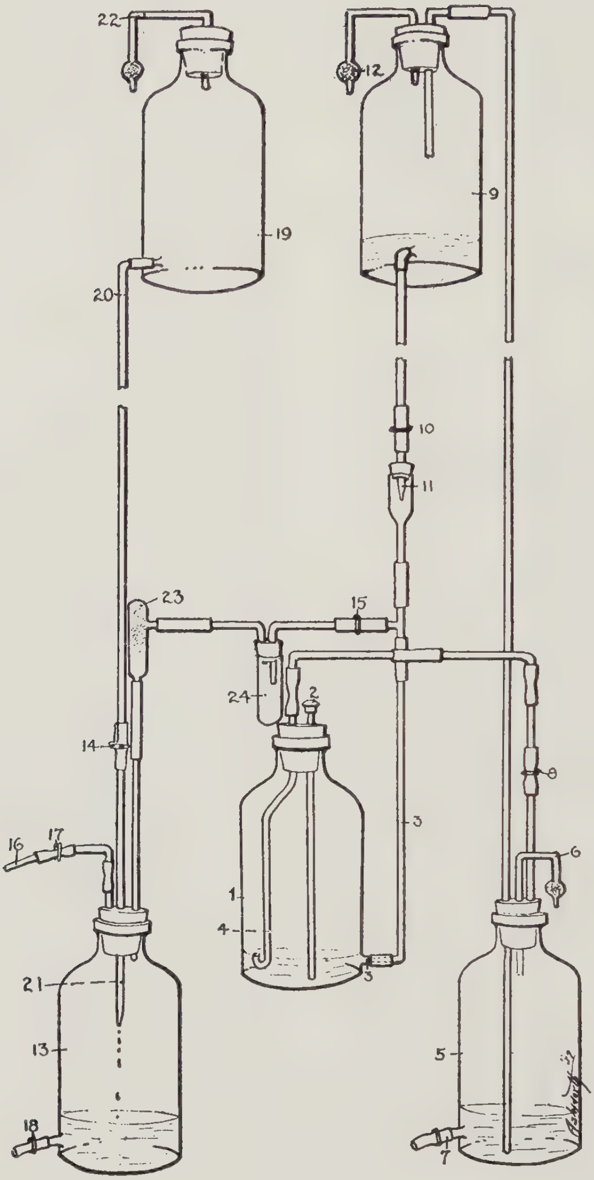
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Fig. 467. Osgood apparatus for bone marrow culture. (From Osgood, E. E.: Am. J. M. Sc. 195:141, 1938.)

Table 281. *Azurophil granules*

Diameter cell in relation to lobocyte	*Size of granules*	*Nucleoli*	*Chromatin structure*	*Shape of nucleus*	*Peroxidase stain*	*Name of cell*
Same or smaller	Coarse	Present or absent	Coarse in clumps	Round or oval; sometimes irregular or cloverleaf	Negative	Lymphocyte
Larger	Coarse	Present or absent	Coarse	Round or oval; rarely irregular or cloverleaf	Negative	Prolymphocyte
				Round or oval; rarely horseshoe	Positive	Progranulocyte A (promyelocyte II)
			Very coarse	Round or oval	Negative	Plasmacyte†
		Present	Fine	Round or oval; rarely horseshoe*	Negative	Lymphoblast or granuloblast
	Fine, diffuse	Present	Fine	Round or oval	Negative	Monoblast
				Horseshoe or irregular	Positive or negative	Promonocyte
		Absent	Coarse clumps and strands	Horseshoe or irregular	Positive or negative	Monocyte
Three times as large	Fine, diffuse	Absent	Coarse clumps and strands	Horseshoe or irregular	Negative	Megalokaryocyte

*Rieder cell.

†Cytoplasm opaque; plasma cells with azurophil granules are very rare.

Table 282. *No granules (all have round or oval nuclei)*

Cytoplasm	*Nucleoli*	*Chromatin structure*	*Diameter of nucleus in relation to diameter of cell*	*Size of cell in relation to neutrophil lobocyte*	*Peroxidase stain*	*Name of cell*
Opaque	Absent	Pyknotic	Less than ½	Smaller	Negative	Metakaryocyte*† (normoblast)
		Coarse	Less than ⅔	Smaller	Negative	Karyocyte*† (pronormoblast)
			More than ⅔	Same or larger	Negative	Prokaryocyte* (erythroblast)
			Less than ½	Usually larger	Negative	Plasmacyte†
	Present	Coarse	Less than ½	Usually larger	Negative	Proplasmacyte
		Fine	Less than ⅔	Usually larger	Negative	Plasmablast
			More than ⅔	Same or larger	Negative	Karyoblast* (megaloblast)
Transparent	Present	Fine	More than ½	Usually larger	Negative	Lymphoblast, monoblast, granuloblast (myeloblast)
	Present or absent	Coarse	More than ½	Larger	Positive	Progranulocyte A (promyelocyte II)
					Negative	Prolymphocyte
				Same or smaller	Negative	Lymphocyte

*May or may not contain hemoglobin; other nucleated cells never contain hemoglobin.

†Sometimes two or more nuclei in one cell.

Osgood found that in cultures of human blood by a technic which with marrow permits multiplication, maturation, and functional activity of cells of the granulocyte series for at least 6 wk. the duration of life of the neutrophils averaged 61 hr. and in the majority of instances was between 48 and 90 hr. It seemed probable that these figures represented about the actual duration of life of these cells in the blood. The duration of the life of eosinophils, from 8-12 days, and basophils, from 12-15 days, determined by this method is much longer than the duration of life of the neutrophils.

In Table 281 from Osgood's article are collected the facts regarding the cells with azurophil granules. Table 282 from Osgood's article shows the facts relating to the cells with no granules.

In Table 282 are shown the facts relating to cells with no granules.

Chapter 68

Spleen and liver in hematologic diseases

JOHN D. BAUER

SPLENIC TUMOR AND HEMATOLOGIC DIAGNOSIS

Enlargement of the spleen is quite common. It is important to classify it, if possible, from clinical and hematologic standpoints.

Summary of causes of splenic enlargement

The causes of splenic enlargement may be grouped under the following headings: (1) mechanical obstruction, in other words, venous congestion, (2) infective and toxic conditions, (3) blood diseases, (4) reticuloses and reticuloendothelioses, with or without disturbance of lipoid metabolism, and (5) hemorrhages.

Venous or passive congestion of spleen

This may occur rapidly from failure of the right heart or from thrombosis or thrombophlebitis of splenic or portal veins. It may occur slowly from some central cause such as mitral stenosis or incompetence either alone or associated with changes at the tricuspid orifice, adherent mediastinopericarditis, chronic fibroid myocarditis, emphysema of the lung with chronic bronchitis, interstitial pneumonia, or adherent pleurisy.

Additional **local** causes are (1) cirrhosis of the liver, (2) tumors of the liver, (3) chronic inflammatory thickening along the portal tracts, either syphilitic or tuberculous, (4) pressure upon splenic vein by slowly growing carcinoma of the head of the pancreas, (5) enlarged lymph glands in the hilum of the liver, and (6) aneurysm.

Enlargement due to acute and chronic infective and toxic conditions

Acute infective and toxic conditions produce an enlarged spleen—hyperemic, proliferative, and infiltrative. This acute splenitis on cut section shows the pulp coming off on the knife, the diffluent spleen.

Chronic infective enlargements are due to tropical diseases, malaria and kala-azar, or to syphilis and tuberculosis.

This initial hyperemia is replaced by hyperplasia of the splenic tissue, especially of the pulp, so that the color is frequently paler than normal, the consistency is either normal or a little increased, and the capsule is thickened. Later hyperplasia of the stroma occurs, with thickening of the organ. The sheaths of the vessels and sometimes the trabeculae show hyperplasia of connective tissue. This may cause atrophy of the cellular constituents in the pulp. Cut sections show distinct mottling with lighter and darker red parts. At times there is marked pigmentation, and frequently there is adherent perisplenitis.

Spleen in malaria: There are found acute inflammatory swelling of the spleen and melanin and hematogenous pigment contained in free or fixed phagocytes or lying free in the sinuses. Parasites are found in the red blood cells of the pulp and in the large phagocytes. In chronic malaria, the spleen is harder from an increase of the reticulum, which predominates over the cellular hyperplasia; the color is gray brown.

Spleen in kala-azar: Enlargement is associated with marked anemia, leukopenia, skin hemorrhages, local dropsies, and enlargement of the liver and exceeds enlargement due to malaria. Microscopically many large mononuclear histiocytic phagocytes, 4-5 times the diameter of a red blood corpuscle, are seen containing numerous oval parasites. Other cells of smaller size lying in the pulp contain red blood cells and pigment. In later stages there is considerable increase of the reticulum, especially near the malpighian bodies.

Tuberculosis of spleen: This is secondary, or there may be tubercles from acute miliary tuberculosis or subacute and chronic tubercles with caseation. In children a bovine origin of the infection is suggested.

Spleen in syphilis: In acquired **syphilis** the spleen is enlarged, especially in the tertiary stage, with waxy or amyloid infiltration. In congenital syphilis in children enlargement is associated with recent or old perisplenitis, accompanied by diffuse fibrosis, endarteritis, and occasionally periarteritis, numerous spirochetes can be demonstrated.

An increase of the reticulum is seen in Hansen's disease, with enlargement, perisplenitis, atrophy of malpighian bodies, and disappearance of lymphocytes. There are numerous leprous cells in clusters.

Splenic enlargements in blood diseases

The spleen is usually greatly enlarged in chronic myelogenous and lymphatic leukemia, in polycythemia and erythremia, in hypersplenism (splenic anemia), and in congenital spherocytosis and thrombocytopenic purpura. Moderate splenomegaly may be seen in hemolytic anemias, pernicious anemia, acute leukemias, infectious mononucleosis, and lymphomas.

Splenomegalies in reticuloses and reticuloendothelioses

This includes active hyperplasia of the true reticulum, with changes in follicles of lymph glands and in malpighian bodies of the spleen. In reticuloses the spleen may weigh as much as 4.7 kg. Red pulp is described with large malpighian bodies, the ordinary lymphocyte being crowded out. Large cells are seen.

Spleen in Hodgkin's disease: Enlargement is uniform, and the capsule is thickened. Section shows congestion and is a brownish red color; malpighian bodies are prominent. There is an accompanying alteration of the lymph glands of the body. Reed-Sternberg cells with nongranular cytoplasm and convoluted, indented, or ring-shaped nuclei, closely resembling the giant cells of bone marrow, are present. Eosinophils are present.

Reticuloses associated with disturbance of lipoid metabolism

Gaucher's disease: The spleen may weigh from 3000-4000 gm. Pulp is infiltrated with Gaucher cells. In the consideration of Gaucher's disease, one must bear in mind that it belongs to the diseases known as xanthomatosis or a series of clinical and anatomic conditions in which lipoid substances are deposited in certain tissue systems or organs of the body. Xanthomatoses have been classified as generalized or local.

There are 2 generalized types—symptomatic and essential or primary.

The symptomatic form is seen as multiple xanthomas in diabetes or liver diseases which are associated with jaundice and also in chronic kidney disease. These xanthomas occur as yellow pealike nodules in the skin. Lipid is deposited in proliferating cells of the connective tissue of the skin and in the endothelium of lymph vessels. These large cells contain fat.

The second type of the generalized form, the essential or primary xanthomatoses, comprises Gaucher's disease, Niemann-Pick disease, Hand-Schüller-Christian disease, and the primary nonsymptomatic external and internal xanthomatoses in the skin and inner organs.

Gaucher's disease was first described by Gaucher in 1882. He called it a primary epithelioma of the spleen. In 1900 Bovaird of New York called it a nonneoplastic disease, stating that it was endothelial hyperplasia in the spleen, liver, and lymph nodes. In 1907 Schlagenhaufer called it a systematic affection of the reticulum cells of all lymphatic and hematopoietic organs, including the bone marrow. In 1907 Marchand recognized the importance of the deposit of foreign substances in the histogenesis of the Gaucher cells. In 1916, Mandelbaum and Downey found conception of the origin of these cells. In 1924, Lieb and Epstein by study of the spleen showed the lipoid nature of the Gaucher substance, showing that kerasin is its important constituent. Kerasin belongs to the cerebrosides (sphingogalactides). This disease is not rare. Pick in 1926 collected 39 cases to that date. Cushing and Stout in the same year collected 50 cases in the United States. Hoffman and Makler[870] collected 80 cases in all, including their own case.

The disease is of familial incidence. Collier first reported its occurrence in more than one member of a family. It is more common in the female.

It is characterized by splenic and liver tumors. The oldest patient of Pick was 56 yr. The bone was involved as noted by pain in the lower ends of the femur and tibia or tenderness over the sternum and ribs. The earliest signs are the characteristic pigmentation of the skin and definite blood changes. The skin pigmentation, confined mostly to the face, neck, and hands, is yellow brown to ochre. There also occur brownish yellow wedge-shaped thickenings of the sclerae, the base of the wedge lying toward the margin of the cornea.

Pathologic anatomy: A very large spleen weighing as much as 8100 gm. is characteristic. Cut section shows a reddish gray, pink, or sometimes chocolate-brown background stippled with innumerable gray-white or gray-yellow markings, corresponding to accumulations of Gaucher cells. Small or large nodular cavernous foci form in the spleen and are always multiple. The liver may weigh 3300 gm. Remember, the normal weight is about 1500 gm.

The lymph nodes show no enlargement of the superficial glands, but enlargement and pigmentation of the intrathoracic and intra-abdominal glands occur.

The essential histologic change is the presence of many Gaucher cells which are demonstrable by acid fuchsin stain after picric acid fixation. The cytoplasm is wrinkled, giving a vacuolated appearance. There are arterial branches within the nests of Gaucher cells. Pigment is absent only in children and infants. Because of the disappearance of Gaucher cells, we see fibrotic nodules. In the liver the process is cirrhotic and contains nests of Gaucher cells.

The peculiar appearance of Gaucher cells is due to the presence of kerasin, which may be demonstrated chemically even in dried spleens. The formula of kerasin given by Rosenheim is $C_{47}H_{91}NO_8H_2O$. It has properties similar to phrenosin and accompanies phrenosin in tissues of the nervous system, both constituting a cerebroside or glycolipin. Capper et al.[871] report a case of Gaucher's disease with presentation of a table differentiating the lipoid disturbances, as shown in Table 283.

Method for demonstration of kerasin (Bloom and Kern[872]) is carried out by taking formaldehyde-fixed spleen or fresh spleen, grinding through a meat chopper, and drying to a constant weight in a vacuum desiccator. The dried powder is then extracted for 5 days with ether in a Soxhlet. The residue is extracted with ethyl alcohol at 40° C. for 36 hr. The material is further extracted at boiling temperature in a fresh portion of ethyl alcohol for 5 days. The ether soluble fraction is treated with acetone, the resulting precipitate of phosphatids is filtered off, washed with acetone, and dried to constant weight in the vacuum desiccator. The acetone and ether are removed from the filtrate, and the residue, containing cholesterol and fatty acids, is dried to constant weight.

That part of the precipitate from the 40° C. alcohol fraction is dissolved in 100 ml. alcohol to which are added 30 ml. cold saturated mercuric chloride. This gives a colorless gelatinous precipitate, much like egg white in appearance, which fills the entire solution. The precipitate is removed by filtration and is suspended in methyl alcohol; hydrogen sulfide is passed through the suspension. The precipitate is filtered off, the filtrate is boiled with charcoal, and is filtered again. This filtrate when concentrated and cooled gives an almost white gelatinous precipitate. Under the microscope this appears as round crystals arranged radially about a nucleus. On hydrolysis of the material for 8 hr. in methyl alcohol and sulfuric acid under a reflux condenser a white crystalline material with a melting point of 56.7° C. is obtained, which corresponds very closely to that of the methyl ester of lignoceric acid.

It is interesting to note that Gaucher cells arise from the endothelium of blood vessels. Gaucher's disease is evidently a primary disease of the reticuloendothelial system, based upon an error in metabolism. The pigmentation is due to the fact that lipid-storing cells of every sort show a tendency to store simultaneously hemoglobinogenic pigments; with stimulation of the reticuloendothelial structures. The mild anemia which is constant in this disease is due to the bone marrow penetration by the Gaucher cells.

The bony changes are demonstrable by roentgenologic examination.

Hematologic findings: The **blood changes** are leukopenia, which may be due to diminution of neutrophils or lymphocytes, moderate hypochromic anemia, often thrombocytopenia, and often bleeding from the nose or gums, hematemesis, melena, or uterine bleeding. Even slightest trauma produces purpuric bleeding. Bone marrow puncture shows a Gaucher type of cell.

Death is not usually due to cachexia but more often to an intercurrent disease, such as pneumonia, sepsis, tuberculosis, or carcinoma. Some of these patients die following splenectomy.

Niemann-Pick disease: Niemann-Pick disease is found in children, especially females, but in contrast to Gaucher's disease its course is rapid and leads to death within 3 yr. Lipoids are found in the spleen. Scar formation in the later stages replaces the lipoid formations. These changes must be distinguished from those occurring in familial amaurotic idiocy, known as Tay-Sachs disease, which may occur simultaneously. The visceral changes in Niemann-Pick disease do

Table 283. *Xanthomatoses or lipoidoses—diseases due to disturbances in lipoid metabolism**

Name	*Type of lipid*	*Blood findings*	*Eyegrounds*	*Spleen, liver, and glands*	*Age*
Gaucher's disease (90-100 cases)	Cerebroside (kerasin A sphingogalactid)	Hypochromic anemia Leukopenia Thrombopenia Later, hemorrhagic diathesis	Negative	Spleen markedly enlarged Liver enlarged Slight or no lymphadenopathy	Infancy to adult life
Niemann-Pick disease or lipoid histiocytosis (Bloom)	Phosphatids	Moderate secondary anemia Slight leukocytosis Vacuolization of nongranulated cells Excess of lipids in blood	Cherry-red spot in macula lutea *occasionally* found	Spleen and liver very large Moderate lymphadenopathy	Infancy
Hand-Schüller-Christian disease (45-50 cases)	Cholesterol and its esters	May be hypercholesterolemia	Negative	Not enlarged	Any age
Tay-Sachs disease or familial amaurotic idiocy	A prelipid	Negative	Cherry-red spot in macula lutea *usually* found	Usually negative	Infancy
Xanthomatoses of skin, etc.	Cholesterol and its esters	Negative	Negative	Negative	Any age

*From Capper, A., Epstein, H., and Schless, R. A.: Am. J. M. Sc. **188**:84, 1934.

not occur in Tay-Sachs disease, nor do all cases of Niemann-Pick disease show the cerebral changes of Tay-Sachs disease, in which the brain is hard and leathery, the fissures are widened, the corpus callosum is atrophied, and fat granule cells collect about the blood vessels.

This disease is similar to Gaucher's disease from a histologic standpoint. It is a typical disturbance of the histiocyte apparatus. There is great enlargement of the liver, a change which occurs much more rapidly than it does in Gaucher's disease.

The blood picture is similar to that of Gaucher's disease.

Anatomically, Niemann-Pick disease differs from Gaucher's disease in that the characteristic cells are much more prevalent in the spleen, liver, lymph glands, and bone marrow. Reuben maintains that the anemia of Niemann-Pick disease is not as severe as that of Gaucher's disease, nor is the leukopenia as pronounced. He also states that the qualitative leukocytic blood picture is unchanged. Moschokowitz reported a case with 33% hemoglobin, 1,600,000 red cells, and 6500 white cells, of which 90% were neutrophils. According to Reuben the prognosis in children is more severe than in adults, and it is a more rapidly fatal disease. It seems to be associated with delayed development of the blood.

Xanthogranulomatosis: Xanthogranulomatosis is also known as the Hand-Schüller-Christian disease. This disease attacks males and is often associated with exophthalmos and diabetes insipidus. Deposits of cholesterol esters within large phagocytic cells fill multiple defects in the skull; there are also deposits in the white matter of the brain and cord, in the periosteum of other bones, and in the lungs, pleura, liver, lymph glands, bone marrow, and spleen.

Banti's disease: The term splenic anemia does not refer to any specific disease; it is simply a term used to describe those cases with both enlargement of the spleen and anemia.

Splenic enlargement may occur in the following conditions: (1) anemias, (2) hemorrhagic diatheses, (3) polycythemia, (4) lymphatic leukemia or lymphogranuloma, (5) myelocytic leukemia or aleukic myeloses, (6)

Bones	*Pathognomonic features*	*Skin*	*Treatment*	*Remarks*
Mottling and rarefaction with cortical thinning; flasklike appearance ends of femora	X-ray appearance of long bones, especially femora Splenic puncture Bone-marrow examination	Subicteric pigmentation of exposed parts in 45%; yellow thickened pingueculae in eyes in 15%	Splenectomy (fatal in 20%) or radiation (both palliative)	Common in Jews
Any bones or organs may be invaded by foam cells	Splenic puncture	Brownish yellow discoloration	No treatment; all fatal	Mental retardation may be present resembling Tay-Sachs disease; most common in Jews
Membranous bones involved	Defects in membranous bones Diabetes insipidus Exophthalmos Gingivitis and stomatitis Adiposogenital dystrophy	Negative	X-ray treatment of involved area causes prompt local healing but does not stop the progress of disease	
Not involved	Eyegrounds Mental retardation Hyperacusis	Negative	All fatal	Brain shows prelipid deposits in granular layer and about dendrites of Purkinje cells; most common in Jews
Any bones may be involved		Xanthomatoses of any part of skin	Excision if tumorous	

chronic infections such as congenital syphilis, malaria, leishmaniasis, etc., (7) rickets in first year of life; later it disappears, (8) acute infections such as typhoid fever, miliary tuberculosis, and septic infections, (9) Gaucher's disease and Niemann-Pick disease, (10) localized splenic tuberculosis, (11) Banti's disease in which there is fibrosis of the spleen, liver cirrhosis, and finally anemia, (12) splenic tumors from circulatory disturbances such as cardiac stagnation in beginning heart failure, beginning heart failure from valvular disease, stagnation from acute or chronic thrombosis in the venae cavae, or through stagnation by reason of pericardial adhesions, and (13) splenic tumor in liver cirrhosis.

Banti's disease is accompanied by enlargement of the spleen without any change in the lymphatic glands, followed by symptoms of simple anemia and by weakness, dyspnea, etc. Hemoglobin content may drop to 30%. There follows a second period during which the liver enlarges without icterus and without ascites, but the splenomegalia remains. This lasts perhaps for 8-13 mo. The third period is accompanied by ascites and decrease in the size of the liver. There is atrophic cirrhosis in the liver. It occurs after hemorrhages or through the progressive liver atrophy. The number of red cells is not markedly decreased, but the color index is lower. Nucleated red cells are not seen. The lymphocytes are usually decreased. Histologic investigation of the organs in fatal cases of Banti's disease show fibroadenosis of the spleen, including the pulp as well as the follicles. In the second stage of the disease there is a sclerotic condition of the intrahepatic veins and later hypertrophy of the interstitial material. No siderosis is seen in the liver.

Blood picture: There is marked and constant leukopenia; sometimes the white cells are decreased to as low as 1000/c.mm. There is no change in the differential picture. As noted previously, the anemia progresses with the advance in the disease. There is a low color index. There may be as few as 3,000,000 red cells/c.mm. with 40% hemoglobin. Hemorrhages into the stomach are followed by further reduction in the red cells and hemoglobin percentage, together with temporary posthemorrhagic leukocytosis. The anemia itself may be severe enough to be fatal. There is an increased resistance of the red cells to hemolysis in hypotonic salt solution.

Splenomegaly in presence of hemorrhages

This enlargement and the edematous softening of the spleen occur whenever there have been blood effusions and hemorrhages of any considerable size in any organ or cavity of the body. It is due to increased function or blood destruction in the main.

Tumors of spleen

Primary fibroma, chondroma, osteoma, lymphangioma, hemangioma, and melanoma of the spleen have been recorded. Of secondary tumors, sarcomas occur less infrequently than carcinomas. The most common type is melanotic sarcoma.

Animal parasites

Most frequent are the hydatids of *Echinococcus granulosus.*

Cysts

Cysts of the spleen are rare. True cysts come from dilatation of blood spaces, cavernous angioma, or dermoids, or they may be parasitic. False cysts may be traumatic due to necrosis with softening, or tuberculous.

Abscess

Abscess of the spleen is seen in sepsis.

SPLENIC PUNCTURE[873]

Splenic puncture requires withdrawal of splenic pulp without blood. Use an oxidation-proof needle of steel or nickel 8-10 mm. diameter and an absolutely air-tight syringe.

Produce superficial anesthesia by means of an intracutaneous Novocain wheal. Insert the needle into the superficial muscular plane strictly in the zone of splenic dullness, immobilize the patient by asking him to hold his breath, give a short, but decided thrust, and rapidly and energetically aspirate, permitting the piston to return into its position of rest before the needle is withdrawn. The patient remains in bed for 12-24 hr. Ice is constantly applied to the splenic region to prevent internal hemorrhage.

Splenograms are made by staining according to Wright-Giemsa (p. 1143), studying the aspect of the red cells and the relative number of hematoblasts. The normal percentages are given in Table 284.

The splenogram shows closer and more numerous cells than the corresponding hemogram. Abnormal erythrocytes are lacking. Platelets are present in larger numbers than in the peripheral blood. Determination of their number and coloration is one of the chief elements of splenic puncture.

Table 284. *Splenogram*

Neutrophilic polynuclear cells	20-30%
Eosinophilic polynuclear cells	1%
Basophilic polynuclear cells	Rare
Typical lymphocytes	50-60%
Medium mononuclear cells	10-20%
Monocytes	5-10%
Plasmacytes	1- 2%
Normoblasts	20%
Naked nuclei	Numerous

Background, thick, sometimes granular; erythrocytes, normal; platelets, very numerous.

Typical splenograms for each of the various pathologic reactions of the spleen are as follows.

Lymphomatosis: The splenogram of the lymphocytic leukemias is remarkable for homogeneity, achieved by the abundance of white elements, expressing the hyperplasia. Adult and young lymphocytes stand out from a clear and also homogeneous background. The young lymphocytes are much more numerous than in a normal splenogram (30-50% instead of 0.5-3%); however, the presence of all intermediary ages between a lymphoblast and a lymphocyte makes it difficult to perform an accurate count. Polynuclear monocytes and plasmacytes and especially normoblasts and megaloblasts are scarce, the latter even exceptional.

The uniformity and constancy of the microscopic appearance of material obtained by splenic puncture in the lymphomatous diseases are a powerful argument for uniting forms apparently so far from each other as lymphocytic leukemias and pure splenic lymphomas.

Myelomatosis: The splenogram of the myelomatoses is marked by a variegated aspect which is due to the great changes of the elements of the myeloid series under the influence of age and also to the more or less marked, but always noticeable, prevalence of a normomegaloblastic, exceptionally megakaryocytic reaction. This notwithstanding, the myelomatous splenogram is a distinct and isolated type. The characteristic cells abound: myelocytes of all ages and all sizes, metamyelocytes, promyelocytes, primitive cells, hemohistioblasts, hemocytoblasts, polynuclears, and erythroblasts. The background is thick and granular, containing numerous platelets and poorly colored red blood cells.

Leukoblastoses: The splenogram of acute leukemia shows a marked prevalence of the typical primitive cells (90-95%). The other cells are more mature yet decidedly young. These elements permit tracing of the origin of the affection—lymphomatosis, myelomatosis, or erythroblastomatosis.

Cryptoleukemias: A new chapter on leukemias, namely, one on atypical leukemias and cryptoleukemias (aleukemias or subleukemia) was written in terms of splenic puncture. These conditions were not capable of diagnosis by the ordinary methods of investigation. Whether or not the peripheral blood shows abnormal elements, the splenogram shows atypical young cells.

RELATIONSHIP OF LIVER TO BLOOD DISORDERS[874]

The liver participates in blood disorders by virtue of its function as a reticuloendothelial organ, its role in the metabolism of blood pigments, in the storage of iron, in the synthesis of blood proteins, and in the storage of the antipernicious anemia factor. Furthermore the spleen, which participates in so many blood dyscrasias, bears an intimate relationship to the liver both physiologically and pathologically.

In most blood dyscrasias the liver is affected to some extent, and it may be markedly enlarged. However liver function is seldom significantly impaired. Green and Conner in 1926 and other investigators since then have shown that all of the commonly used liver function tests usually give normal results in hepatomegaly due to leukemia, polycythemia, hemolytic jaundice, pernicious anemia, and even Gaucher's disease. In polycythemia the liver is frequently enlarged due to chronic hyperemia, in leukemia there may be marked infiltration of the liver with leukemic cells, in pernicious anemia the hepatomegaly which is sometimes seen is due to fatty changes and hemosiderin deposits, whereas in Gaucher's disease, Hodgkin's disease, lymphosarcoma, and several other diseases of the reticuloendothelial system striking pathologic changes may be found in the liver. The fact that these diseases rarely result in any significant impairment of liver function either clinically or by laboratory testing is a manifestation of the great reserve power of the liver.

Jaundice is common in the blood dyscrasias, and this sometimes leads to an incorrect diagnosis of primary hepatic or biliary tract disease. In congenital or acquired hemolytic icterus, the jaundice is obviously due to the rapid breakdown of red blood cells either as a result of an inherent fragility of the erythrocytes or possibly due to the action of a hemolysin. In pernicious anemia the characteristic lemon yellow color is due to either an abnormality of pigment metabolism or hemolysis.

Rich and associates have suggested that in any hemolytic anemia the jaundice may be due to a combination of rapid disintegration of erythrocytes with a hepatic lesion. It is very difficult to produce jaundice in a dog by the injection of bilirubin. Furthermore, Rich showed that simple hemorrhage in dogs as well as pernicious anemia and secondary anemias in human beings may be accompanied by damage to the cells around the efferent veins of the liver lobules. This lesion is identical with that seen in chronic passive congestion of the liver, and Rich postulated that the lesion is due to tissue anoxia resulting from the anemia. The jaundice is thus due to a combination of hemolysis plus functional liver damage. In human hemolytic anemias a hepatic lesion is not always demonstrable histologically, but it is probably true that a perfectly normal liver can excrete all the bilirubin the body is capable of producing; hemolysis alone, therefore, probably cannot produce jaundice unless the liver is functionally deranged.

In leukemia, as well as in the so-called lymphomas, mild jaundice sometimes occurs due to infiltration of the liver or destruction of liver cells by the lymphomatous process. Occasionally enlarged lymph nodes in the portal fissure may occlude the bile ducts and produce marked jaundice. Jaundice due to this mechanism may be completely relieved by x-ray therapy directed to the upper abdomen.

Hemorrhagic disorders due to diseases of liver or biliary tract: In any hemorrhagic disease there is always a defect of either capillary permeability or the coagulation mechanism. So far as is known, the liver has nothing to do with capillary permeability, but the liver does contribute to blood coagulation. There are 4 essential substances which must be present in order for blood to coagulate normally: thromboplastin, Ca ions, prothrombin, and fibrinogen. Of these, fibrinogen is probably synthesized solely in the liver, whereas prothrombin is synthesized primarily in the liver. Thus theoretically liver disease may result in hemorrhage due to failure of synthesis of either of these factors.

Actually, a condition known as **congenital fibrinogenopenia** has been described in which there is a specific congenital defect in the ability of the liver to synthesize fibrinogen; however, only half a dozen cases have been described. The amount of fibrinogen normally present in the plasma is far in excess of what is required for coagulation. In any kind of severe liver disease, the plasma fibrinogen level may be decreased below normal, but this is always overshadowed by the much more marked and more significant decrease in prothrombin.

One of the factors that may produce bleeding due to **inadequate amount of prothrombin in the blood is vitamin K deficiency.** Dietary deficiency never produces vitamin K deficiency in adult human beings, as proteolytic bacteria in the intestines can synthesize all the vitamin K that is required. However during the first few days of life there are few if any bacteria in the intestines, and in the newborn infant prothrombin deficiency is common. This is generally accepted as being the principal factor responsible for hemorrhagic disease of the newborn.

In adults, vitamin K deficiency occurs only when there is poor absorption from the intestinal tract due to either severe diarrhea or absence of bile salts in the gut. Vitamin K is fat soluble and is not absorbed in the absence of bile salts. Thus, the bleeding which occurs in severe obstructive jaundice can be corrected either by the parenteral administration of vitamin K or by the administration of bile salts by mouth.

In contrast to this type of prothrombin deficiency, the **prothrombin deficiency resulting from severe liver damage** is not affected by vitamin K administration. When the liver cells which synthesize prothrombin are destroyed, the only rational therapeutic approach is to keep the patient alive by supplying prothrombin from without in the form of plasma transfusions. This stops the bleeding only temporarily but is an extremely important procedure whenever the nature of the liver disease is such that regeneration of liver tissue is possible.

A condition known as **congenital hypoprothrombinemia** has been observed in a few children in which the liver lacks the ability to synthesize prothrombin. There have been very few cases of this sort reported in the literature, but now that it is being looked for, it is being recognized more frequently. Quick stated that he has knowledge of over half a dozen such cases himself.

Role played by liver in certain anemias: The liver plays an important part in 2 types of anemia—pernicious anemia and iron-deficiency anemia. It is well established that erythrocyte maturation factor is stored in the liver, and the liver of patients with pernicious anemia lacks this substance. It has been shown that cirrhotic livers are deficient in erythrocyte maturation factor. It is, therefore, very easy to assume that the anemia so commonly seen in various types of liver disease is due merely to the absence or diminished quantity of this factor in the liver.

It is known that some degree of anemia is usually present in advanced cirrhosis of the liver. But perhaps sufficient emphasis has not been placed on the fact that in only about half of these cases is the anemia macrocytic. The mean corpuscular volume in general is in the vicinity of 100 c.μ; however, the macrocytosis does seem to increase as the anemia becomes more marked. The anemia of cirrhosis is often accompanied by reticulocytosis and occasionally by mild leukopenia. It has frequently been suggested that because of the close resemblance of the blood picture to that of pernicious anemia, the damaged liver is responsible for a deficiency of antipernicious anemia factor. A reticulocyte response to the administration of liver extract does occur in some of these cases, but in other cases of cirrhosis no such effect is obtained. It is probable that the anemia of cirrhosis and other types of severe liver disease is not due to deficiency of erythrocyte maturation factor.

It is important to remember that macrocytosis which is so characteristic of pernicious anemia also occurs in the anemias of chronic uremia and occasionally in leukemia. Furthermore in normal animals the kidney as well as the liver contains large amounts of antipernicious anemia factor, and thus it is hard to see how liver disease alone could produce a significant decrease in the amount of available erythrocyte maturation factor unless the kidneys were simultaneously damaged.

Castle studied the macrocytic anemias due to nutritional deficiency. In 2 of the 3 patients he studied, the anemia developed during pregnancy. In spite of the fact that the anemias in all 3 of these patients were strikingly macrocytic, no reticulocyte response occurred in any of them following the intramuscular injection of liver extract. In all 3 cases, however, a reticulocyte response followed by a gradual rise in red blood cell counts occurred when liver extract was given by mouth. Castle concluded that the "unitarian" hypothesis which regards all macrocytic anemias as being due to deficiency of erythrocyte maturation factor is not tenable. He suggested that in this type of nutritional macrocytic anemia the digestive organs form a new hematopoietic substance from the liver extract in a fashion analogous to the food-gastric factor relationship in true addisonian pernicious anemia. Present evidence does not justify the conclusion that all macrocytic anemias are due to the same mechanism; there is no conclusive proof that the macrocytic anemia of liver disease is due to deficiency of antipernicious anemia factor.

When red blood cells are hemolyzed in the body, the hemoglobin is broken down, and the iron thus liberated is stored in the reticuloendothelial tissues throughout the body. The liver is perhaps the most important of all the organs which store iron. By virtue of this storage mechanism, extremely minute quantities of iron are lost from the body by excretion. It is for this reason that hypochromic microcytic anemia due to iron deficiency practically never occurs except in hemorrhagic states in which large amounts of actual hemoglobin are lost from the body. Hypochromic microcytic anemia due to iron deficiency very rarely if ever occurs as a result of inadequate intake of iron in the diet. Thus the liver plays an important part in iron metabolism.

Chapter 69

Hematology of infants and children

JOHN D. BAUER

Since blood pictures in infants and children normally differ somewhat from those in adults, we shall give briefly the salient differences so that proper interpretation of findings may be made.

At birth the number of white cells is very high, around 30,000, and declines to normal after the first week. It is also interesting to note that there is a left shift during the first week of life. The count of the blood platelets is also variable during the first week, sometimes reaching very high proportions.

The percentage of hemoglobin in blood at birth is very high, figures as high as 130% having been obtained. Hemoglobin falls soon after birth, reaching normal around the end of the first week.

The number of red cells is very high at birth, beginning to fall on the third day after birth.

At birth the presence of nucleated red cells in the film has no pathologic significance. The same is true of the great prevalence of reticulocytes and polychromatic cells. Anisocytosis is common and not pathologic.

One must divide these young subjects into 3 groups in order to get a proper estimation of hematologic data: newborn babies, nursing infants, and infants from 1-6 yr. of age. The average adult blood picture is not reached before the sixth year, but even then and sometimes later lymphatic reactions are produced much more easily than in the adult and wide fluctuations are commonly seen. In general, the blood picture at birth is markedly neutrophilic and shows a great shift to the left. Physiologic myelocytes and juvenile cells have been seen at this time under perfectly normal conditions. The neutrophilia with marked shift, as Arneth says, represents pathologic leukocytosis of the birth process. It is very probable that the accommodation of the body to the new circulation and respiration irritates the residue of embryonal blood forms and liberates them into the blood stream. Lymphocytes increase rapidly in the relative picture.

The blood pictures of prematurely born babies exhibit greater abnormalities than those of normally born children; i.e., their blood pictures may closely approximate the embryonal blood picture. Prematurely born children at first show high leukocytosis and neutrophilia. This recedes, together with the marked nuclear shift.

BLOOD PICTURE OF NURSING INFANTS

The blood picture of the nursling remains constant in the first year and up to the middle of the second year. At this time it begins to change gradually into an appearance like that of the blood of the adult, finally attaining this adult appearance about the sixth year. There is still high **lymphocytosis.** The number of neutrophils in the nursing child is about 25-30%, and the lymphocytes are from 42.5-90.5%, according to Hofmann and Welker. Gundobin considers the following the average for nurslings: neutrophils, 34.6%; lymphocytes, 59%; monocytes, 6.4%.

Morphology of blood in infancy

There is an extreme lability of the blood-forming apparatus in early infancy; i.e., it is much more sensitive to irritation than that of the older individual.

Table 285. *Blood picture in nurslings ages 1-12 mo.**

No. cases	Age (mo.)	Hemoglobin (%)	Red cell count (c.mm.; millions)	White cell count (c.mm.)	Neutrophils (%)	Eosinophil (%)	Basophil (%)	Lymphocyte (%)	Monocyte (%)	Metamyelocyte (%)
9	1	96	5.51	16,130	24	2.0	1.5	60	11.0	0.5
8	2	96	5.5	16,040	24	2.0	1.5	61	10.5	1.0
8	3	96	5.43	13,100	24	2.5	1.0	62	10.0	0.5
7	4	95	5.41	13,000	23	2.5	0.5	62	12.0	–
8	6	95	5.39	12,850	21	2.5	0.5	64	12.0	–
6	8	94	5.36	12,600	24	2.5	0.5	62	11.0	–
7	10	93	5.35	12,140	27	2.5	1.0	60	9.5	–
6	1	95	5.22	16,600	26	2.0	1.0	59	9.5	2.5
6	2	93	5.15	16,430	29	2.0	1.0	57	8.5	2.5
5	4	93	5.14	12,700	32	2.0	0.5	54	10.0	1.5
6	6	91	5.07	12,100	34	1.5	0.5	53	10.0	1.0
7	10	90	5.04	11,540	36	1.5	0.5	53	8.5	0.5
7	12	90	5.01	11,100	37	1.5	0.5	53	8.0	–
8	1	91	5.2	17,210	27	1.5	1.0	56	11.0	3.0
7	2	90	5.14	17,260	30	1.5	1.5	54	10.5	2.5
6	4	88	5.03	12,200	32	1.5	1.5	52	10.5	2.5
7	6	88	4.95	11,600	36	1.5	1.5	49	10.0	2.0
6	10	84	4.9	11.000	40	0.5	0.5	48	9.5	1.0
8	12	84	4.83	10,110	42	0.5	0.5	47	9.0	0.5

*From Baar, H., and Stransky, E.: Die klinische Hematologie des Kindesalters, Wien, 1928, Franz Deuticke, p. 42.

Table 286. *Hemoglobin and hematocrit values in children*

Age	No. of cases	Hemoglobin (gm.)	Vol.%	Hemoglobin (%)	Maximum	Minimum
2-3 wk.	6	17.0	22.8	123	133	108
3-4 wk.	4	15.7	21.1	114	127	110
4-5 wk.	6	14.4	19.3	104	117	87
5-6 wk.	3	13.7	18.3	99	122	77
6-7 wk.	4	12.7	17.0	92	99	85
7-8 wk.	4	12.0	16.1	87	97	77
2-3 mo.	6	10.9	14.6	79	86	75
3-6 mo.	19	11.5	15.4	83	88	70
6-9 mo.	16	11.2	15.0	81	87	70
9-12 mo.	6	11.0	14.8	80	84	74
1-2 yr.	11	11.6	15.5	84	94	72

Table 287. *Hemoglobin content in children*

Age	Perlin	Lichtenstein	Wick	Widowitz	Stierlin	Carstanjen
1- 2 yr.	78-80%	74-78%	–	62-110%	44-78%	70%
2- 3 yr.	80%	75%	–	62-110%	44-78%	76%
3- 4 yr.	80%	77%	83-110%	98%	44-78%	75%
4- 5 yr.	84%	77%	83-110%	92%	44-78%	75%
5- 6 yr.	81%	79%	–	86-110%	80.5 %	75%
6- 7 yr.	85%	80%	–	86-110%	80.5 %	65%
7- 8 yr.	85%	80%	–	86-110%	80.5 %	85%
8- 9 yr.	85%	79%	–	86-110%	80.5 %	73%
9-10 yr.	85%	79%	–	90-110%	80.5 %	70%

Table 288. *Differential counts in children*

Age	Polymorphonuclear cells (%)	Lymphocytes (%)	Mononuclear cells (%)	Eosinophils (%)
1- 6 mo.	34.54	50.78	11.09	3.59
6-12 mo.	40.84	49.21	9.19	0.76
1-2 yr.	41.99	47.0	7.97	3.04

Table 289. *Differential counts in children*

Age	*Neutrophils* (%)	*Lymphocytes* (%)	*Monocytes* (%)	*Eosinophils* (%)
2- 4 mo.	29.1	56.3	11.2	2.8
4- 6 mo.	27.9	56.9	11.1	3.9
6- 8 mo.	28.1	58.3	9.8	3.4
8-10 mo.	28.0	61.2	8.2	2.5
10-12 mo.	33.7	56.2	7.1	3.0
1- 2 yr.	34.0	54.5	6.6	5.0

Table 290. *Differential counts in children*

Age	*Hemoglobin (%)*	*Red blood cell count (millions)*	*White blood cell count*	*Platelet count*
3 wk.	–	5.4	–	392,000
4 wk.	120	5.2	36,300	296,000
7 wk.	–	4.4	–	336,000
2 mo.	85	4.4	6,900	464,000
2 mo.	75	3.9	5,700	568,000
2 mo.	85	3.6	7,600	304,000
9 wk.	75	3.5	14,000	272,000
10 wk.	–	3.1	7,900	280,000
3 mo.	70	3.8	17,500	292,000
3 mo.	60	3.6	20,000	368,000
5 mo.	75	4.2	7,600	304,000
6 mo.	70	4.03	7,800	272,000
6 mo.	75	3.4	3,450	288,000
6 mo.	85	4.8	13,900	408,000
7 mo.	–	5.8	–	516,000
8 mo.	80	5.45	15,600	416,000
12 mo.	80	4.6	11,000	328,000

Table 291. *Differential counts in children**

Age	*Polymorphonuclear cells (%)*	*Lymphocytes (%)*	*Mononuclear cells (%)*	*Eosinophilic leukocytes (%)*
1- 2 yr.	41.99	47.00	7.97	3.04
2- 3 yr.	48.23	38.45	9.42	3.9
3- 4 yr.	52.63	33.2	8.43	5.74
4- 5 yr.	60.98	25.08	7.64	6.3
5- 6 yr.	55.39	31.04	7.35	6.22
6- 7 yr.	57.75	30.28	8.63	3.34
7- 8 yr.	60.58	27.98	7.75	3.69
8- 9 yr.	58.55	27.98	7.94	5.33
9-10 yr.	56.99	28.43	9.05	5.63
10-11 yr.	51.86	33.03	7.80	7.31
11-12 yr.	60.67	28.26	7.97	3.1
12-13 yr.	53.79	33.25	9.39	3.57
13-14 yr.	62.82	25.88	7.14	4.16
14-15 yr.	56.45	28.22	9.47	5.86

*From Carstanjen: Jahrb. f. Kinderhk. **52**:1900.

Many authorities have noted the high percentage of hemoglobin and increase of cell count at birth. These figures fall rapidly after the first few days. Perlin noted a drop in hemoglobin after the fourth day with a gradual decrease, reaching its minimum at the end of the first year, namely, 58-78%. From the second year on it begins to rise, up to the fifteenth or sixteenth year of life.

The count of the red cells drops soon after birth. Toward the end of the first year it is at its lowest point, namely, 4,200,000, when the count begins to rise, up to the end of the fourth year.

Williamson and Appleton[875] give the figures shown in Table 286.

One must be careful in infancy cases in drawing conclusions from the shift of the neutrophils for the reason that 1.5% juvenile cells and 18% stab cells as well as an occasional myelocyte have been found in healthy infants. This is due to the remarkable lability of the hematopoietic apparatus in infancy. **The hemogram of Schilling is useful in infancy but**

not from a single examination. Conclusions must be drawn only from repeated examinations.

The number of blood platelets in infancy varies greatly. The average count is between 200,000 and 300,000. McClanahan and Mitarbeiter[876] in a report on 68 blood examinations in infancy gave the figures shown in Table 290 on blood platelets.

The hemoglobin content of the blood of children from 1-10 yr. of age is seen in Table 287.

Sedimentation phenomenon of the red cells in infancy is different from that in adults. In the Linzenmeier test in adult females, sedimentation drops to 18 mm. in 800-1000 min. and in males in 1200-1400 min. In newborn babies, the sedimentation rate of 18 mm. occurs in from 6-7 hr., but after the first month the rate is 90 min.

BLOOD PICTURE OF PREMATURELY BORN INFANTS

The blood of prematurely born children offers an interesting study. In the first 2 weeks, the average percentage of hemoglobin is 105; in the third week, 93; in the fourth week, 68; in the second month, 55; in the third month, 47; in the fourth month, 50; in the fifth month, 52. The number of red cells begins to decrease after the second month, reaching its minimum in the third month and attaining a normal figure in the sixth month. There is leukopenia in all these babies up to the sixth month. Lichtenstein regards the anemia of prematurely born babies as physiologic.

Frankenstein and Stecher, in a study of the blood picture of prematurely born babies for the first 12 wk. of life, show a lymphocyte average of 72% in the second week and 82% in the fourth week, which drops later to 72%. Segmented forms in the first 5 days are between 20-28%, rising to 35% and dropping to 26% on the eighth day and to 10% in the fourth week. The stabs decrease in the first week from 12 to 4%. Juvenile forms in this period show 0.5-1%.

BLOOD PICTURE OF CHILDREN FROM 1 TO 6 YR. OF AGE

These subjects show a gradual approach of the nursling's blood picture to that of the adult. Note Table 291 of Carstanjen in which are shown changes from the first to the fifteenth year. The decrease in lymphocytes begins definitely about the end of the second year.

CHANGES IN LEUKOCYTIC PICTURE UNDER PHYSIOLOGIC AND PATHOLOGIC INFLUENCES IN INFANCY

Virchow first called attention to digestion leukocytosis. He noted an enlargement of the mesenteric lymph glands after a meal, together with an increase in white cells in the circulating blood. Gregor in 1898 failed to confirm this phenomenon in healthy infants. In 11 cases he found from 3 to 5 hr. after a meal some degree of leukocytosis, but in 6 cases he noted leukopenia. In general, digestion leukocytosis is not constant in infancy. On the contrary there is more likely to be leukopenia. Schiff and Stransky claim that in healthy as well as in artificially nourished infants whether the lymph is disturbed or not after a milk meal leukopenia follows.

No reliable data are at hand as to the shift in the hemogram as a result of the digestion of food.

Wernstedt[877] found the leukocyte count in infants lower while asleep than it was when they were awake. There is lymphocytosis described as a result of efforts in crying, but this disappears after 30 min. of rest. It is probable that the lymphocytes in crying infants and in babies with whooping cough come from the spleen and the thoracic duct. This exercise leukocytosis was first described by Grawitz in 1910.

In intoxications of infants not due to infectious diseases the total white cell count may be elevated up to 20,000. There is a slight shift to the left in such cases. In intestinal decomposition there is a slight shift, and in extreme cases of marasmus there is a definite left shift. In general, however, a left shift speaks for an infection. In acute toxicosis there is leukocytosis.

The significance of eosinophilia in infancy has been the subject of much discussion. In general, eosinophilia is found in all cases of exudative diathesis. This is particularly true of eczemas of infants, but it is believed that eosinophilia is not due to the local process but to the underlying cause of eczema. Figures as high as 65% eosinophils have been noted. Eosinophils in all acute dermatoses are decreased, and they are increased in all chronic cases, particularly in eczema. In erythrodermia desquamativa the leukocyte count is elevated to 12,000 with a high-grade anemia and a count of eosinophils between 1-6%.

LEUKEMIAS IN CHILDREN

Acute leukemia

According to Fraenkel this is a form of leukemia which begins suddenly and has a rapid termination. The first case reported was by Friedreich[878] in 1857. Up to 1889 Epstein had reported 16 cases. Of the 45 cases collected by Theodor[879] in 1897, 9 occurred in children. A monograph of Benjamin and Sluka[880] in 1907 reported 36 cases of acute leukemia in children from the age of 2-14 yr.

These cases in children begin with fever, headache, pains in the legs, weakness, vomiting, and chills. They show a triad of symptoms, anemia, hemorrhagic diathesis, and ulcerative, gangrenous changes in the digestive tract, especially in the mouth. Other workers have described nodular changes in the skin as part of the acute leukemic process. Hemorrhagic diathesis, seen in the form of hemorrhages under the skin and visible mucosae and sometimes rectal hemorrhages, has been reported. Hemorrhage in the cerebrum may lead to hemiplegia. Subperiosteal hemorrhages occur. Swelling around the tibia has been reported. Changes in the central nervous system are seen in the form of polyneuritis.

The lymph glands are enlarged. The spleen is enlarged. The heart is enlarged; there is a small, accelerated pulse. Sometimes hemorrhagic nephritic symptoms are seen. Septic symptoms are noteworthy.

The blood picture in acute leukemia of children shows changes not only in the white cells but also in the erythrocytes and blood platelets. There is a severe progressive anemia combined with subicterus. The red cell count may go below 1,000,000. The hemoglobin does not keep pace with the drop in the red cell count so that the color index is high. Megalocytes and megaloblasts may be seen so that these cases have sometimes been mistaken for pernicious anemia. The number of white cells is so increased that the relativity of white cells to red cells is about 1:100. Wollstein and Bartlett[881] refer to the aleukemic stage of acute leukemia, in which there is marked change in the differential picture but not a great increase in the total number of white cells. In acute leukemia in children, we see large nongranulated single-nucleated cells. These juvenile lymphocytes in the case of acute lymphocytic leukemia are so definite that we can make a diagnosis upon their appearance. In the acute myelocytic leukemias there are many mononucleated cells, the myelocytes of Ehrlich. There are also mononucleated eosinophilic leukocytes. Many mitoses are seen in the white cells. Resort must be had to the Graham oxidase stain to distinguish myeloid from lymphatic cells.

Monocytic leukemia is similar to the leukemia in adults.

Chronic leukemia

Chronic leukemia of children is very uncommon and is confined to chronic myelocytic (granulocytic) leukemia. The spleen enlarges slowly. The lymphatic glands enlarge later. The blood picture is very characteristic. Hemoglobin content and the number of red cells may be normal in the beginning but later decline. Later there are signs of chronic anemia, polychromasia, anisocytosis, and poikilocytosis and presence of normoblasts and megaloblasts.

At times it is necessary to make a differential diagnosis between the blood picture of infectious leukocytosis and that of chronic leukemia in children. In both conditions the count of the leukocytes is increased, but eosinophils and monocytes are low or absent in septic leukocytosis but are present in large numbers in chronic myelocytic leukemia.

Leukemia of nurslings

Congenital leukemia has been described. Its association with chromosomal aberrations has been mentioned previously.

ANEMIAS OF INFANCY AND CHILDHOOD

Anemia is very common in these young subjects. Attention has been called to the discrepancies in the figures of hemoglobin and red cells between infancy and adult ages. The physiologic anemia of premature babies has been considered.

There are a number of hereditary anemias, all hemolytic (discussed in the appropriate sections). These include hereditary spherocytosis, nonspherocytic hemolytic anemia, elliptocytosis, thalassemia, and sickle cell anemia. Erythroblastosis fetalis is also genetically determined.

There are a number of anemias in children and infants which are closely linked to the development of the fetus, the infant, and the young child. Although they fall within the over-all classification of the anemias discussed in previous sections, it is interesting to collect them in one classification as the anemias of infancy and childhood.

- Erythroblastosis fetalis
- Anemias due to
  - Fetal bleeding into maternal circulation
  - Delivery hazards such as abruptio placentae and cesarean section
  - Early tying of the umbilical cord
  - Iron deficiency of the mother
  - Congenital and hereditary factors
  - Iron deficiency aggravating the physiologic iron deficiency
  - Prematurity

In children and infants certain anemias[882] are therefore more common than they are in adults. In early infancy one should consider erythroblastosis fetalis, iron-deficiency anemia, anemia due to blood loss, megaloblastic anemia of infancy, and congenital anemia. In later infancy and childhood the following are added: anemia of infection, aplastic and hypoplastic anemias, and the congenital hemolytic anemias.

Infectious and postinfectious anemia

These anemias follow such infections as congenital syphilis, sepsis, and malaria. In tropical countries, it is often difficult to distinguish between chronic malaria in children and leishmaniasis infantum. In chronic malaria there is an early appearance of fever, gastrointestinal disturbance, spleen and liver edema, and anemia with pallor. In leishmaniasis there is a late development of these symptoms, followed by late fever and gastrointestinal symptoms. In chronic malaria, there is swelling of the liver and spleen, with a sensitiveness of these organs particularly to pressure in the neighborhood of the phrenic nerve. In leishmaniasis there is swelling of the liver and spleen but no sensitiveness. In chronic malaria there are gastrointestinal disturbances more often with constipation than with diarrhea, whereas in leishmaniasis, gastrointestinal symptoms occur but more often with diarrhea than with constipation. The edema in chronic malaria has certain definite localizations, such as the face or the lower extremities, whereas in leishmaniasis the edema is seldom in the face and more often in the lower extremities. In leishmaniasis the blood shows oligemia; erythroblasts are rare. White cells are decreased. There is usually relative lymphocytosis. Resistance of the red cells to hemolysis in hypotonic salt solution is lowered. Parasites may be found in the blood very rarely but can be found in splenic puncture.

Hematoxic anemia

Anemias due to poisons such as lead, potassium chlorate, pyrogallol, etc., are no different in children than they are in adults. There are many cases of lead anemia in infants and children. It is interesting to note that a symptom of lead poisoning in infants is convulsion.

Lead anemia has already been described (see discussion of hematology of lead poisoning). A very important symptom of lead poisoning in children is the deposit of lead in the ends of the growing bones, which can easily be determined by roentgenologic examinations.

Anemia due to intestinal parasites

Severe anemias in children due to intestinal parasites are common. These are seen in connection with infestation with *Diphyllobothrium latum* and *Taenia.* Theodor, Cima, Naegeli, and others have repeatedly called attention to anemias caused by infestation due to *Trichuris trichiura.* Cima, Cozzolino, Ashford, and others have described anemias due to *Ancylostoma duodenale;* and those due to *Schistosoma haematobium* have been described by Kautzky-Bey. Stäubli, van Cott, and others have described the anemias due to trichinosis. Kautzky-Bey claims that *Schistosoma haematobium* and not *Ancylostoma duodenale* is the causative agent of the so-called **Egyptian anemia** in children. Egyptian anemia is characterized by a disturbed relationship between the hemoglobin content and the number of red cells and by the presence of oligochromemia and marked eosinophilia. In trichinosis in children there is at first polycythemia and later slight hypochromic anemia. *Ascaris* and *Enterobius (Oxyuris)* infestations do not often produce anemizing symptoms.

Constitutional anemia

By constitutional anemias is meant those due to some cause other than poisoning or infections. Many of these anemias are due to disturbances of the glands of internal secretion. Some are due to a letdown in the physiologic activity of the hematopoietic organs. In this latter type are seen anemias in connection with the stigmas of congenital defects.

The anemia connected with internal glandular secretory function is seen particularly in myxedema. Here is seen oligocythemia, oligochromemia, relative lymphocytosis, leukopenia, and often eosinophilia. The blood picture is also characteristic in myxedema, namely, lymphocytosis and neutropenia.

Pernicious anemia

True pernicious anemia is seldom if ever seen in children. Most cases are really pernicious anemia blood pictures due to infestations by worms. It is generally conceded that children are not disposed toward pernicious anemia.

Aplastic anemia

Aplastic anemia was first described by Ehrlich in 1888. It has also been called panmyelophthisis of Frank and aregenerative anemia of Pappenheim. For fuller details, see the discussion of aplastic anemia in adults. Eppinger has divided this anemia into 2 classifications—acute and chronic. Typical cases of aplastic anemia are very rare in children. Most so-called aplastic anemia in children is really aleukemic leukemia with aplastic blood pictures.

HEMORRHAGIC DISEASES OF CHILDREN

Basically the mechanisms involved in hemorrhagic diseases in children are similar to those in hemorrhagic diseases in adults. Blood must be obtained by skin puncture, and the test must be modified to allow use of small blood volumes.[883]

Chapter 70

Normal blood findings in domesticated animals

JOHN D. BAUER

The use of various animals for laboratory purposes requires some knowledge of the normal blood picture of these laboratory and domesticated animals.

Guinea pigs: Guinea pigs have an average number of red cells of 5,250,000. The hemoglobin varies from 85-100%, with an average of 94%. The total number of leukocytes varies from 5000-22,000, with an average of 11,000-12,000. Guinea pigs have an average of 350,000 blood platelets/c.mm.

Rabbits: In rabbits the number of red cells varies from 5,000,000-8,000,000,with an average of 6,000,000. Average hemoglobin value is 96%. The number of leukocytes ranges from 8000-13,000, with an average of 11,000. The differential picture is lymphocytes, 45-55%; monocytes, 2-8%; polymorphonuclear cells, 40-50%; eosinophils, 0.5-1%; basophils, 4-8%. An average reticulocyte count of 4-6% is noted.

Cats: Total number of red cells is about 6,000,000. Goodall[884] found the normal to vary from 7,280,000-8,600,000 with an average of 8,000,000. There are 7000-19,000 leukocytes, with an average of 13,300. Goodall found higher numbers of leukocytes. The differential picture is monocytes, 37%; neutrophils, 54%; eosinophils, 9%; mast cells (basophils), 1%.

Dogs: Red cells range from 5,000,000-8,000,000, with an average of about 6,000,000. Hemoglobin values vary from 55-104%, with an average of 90%. Different investigators have reported different quantities of leukocytes in dogs. Six thousand to 12,000 with an average of about 8000 seems to be the normal for adult dogs. The differential picture is lymphocytes, 11-29%, average, 20%; monocytes, 3-10%, average, 6%; neutrophils, 60-76%, average, 68%; eosinophils, 1-10.5%, average, 6%; with basophils very rarely found.

Goats: Red cells are 9,000,000-10,000,000/c.mm. Leukocytes are around 10,000/c.mm.

Sheep: Average number of red cells is 8,000,000 and of leukocytes is 8000. Amount of hemoglobin varies from 47-63%. The percentage of white cells in the differential picture in sheep is lymphocytes, 40-60; monocytes, 3-11; neutrophils, 30-55; eosinophils, 0.2-0.8; basophils, 0-2.

Cows: Red cells vary from 5,000,000-8,000,000, with an average hemoglobin range of 45-85%. Normal number of leukocytes is 5000-10,000. Differential picture, according to Dimock and Thompson,[885] is lymphocytes, 54.2%; monocytes, 1.4%; neutrophils, 30.5%; eosinophils, 13.15%; basophils, 0.59%.

Horses: Normal number of red cells for stallions is from 7,000,000-10,000,000, with an average of 8,000,000; for geldings 5,500,000-9,000,000, with an average of 7,500,000-8,000,000; for mares, 5,500,000-7,500,000, with an average of 6,500,000-7,000,000. The hemoglobin varies from 85-100%. Leukocytes vary from 5000-10,000, with an average of 8000. The differential picture is lymphocytes, 34-38%; neutrophils, 60-65%; eosinophils, 1-2%.

Swine: Normal count of red cells is about 8,000,000, slightly less for young pigs. Hemoglobin values are around 85%. Leukocyte counts are 19,000 with more than 50% of them lymphocytes. The differential picture of swine shows lymphocytes, 56.4%; neutrophils, 38%; eosinophils 5.13%.

White rats: Total blood vol. about 50 ml. The erythrocytes normally show anisocytosis and polychromasia. The polymorphonuclear cells are hypersegmented. The red blood cell count is 8,500,000.

White mice: Hematocrit is 43 vol.%. Normally anisocytosis and polychromasia are present. The red blood cell count is 9,000,000.

Frogs: Large oval nucleated red blood cells are characteristic. The hemoglobin is 60%, and the red blood cell count is 380,000.

Chapter 71

Summary of quantitative changes in blood cellular elements and hemoglobin

JOHN D. BAUER

RED BLOOD CELL CHANGES

Some conditions with high red blood cell counts

Physiologic:

Epinephrine excretion in certain emotional conditions
Compensatory at times in anemia (chlorosis) and in leukemia under x-ray treatment
Certain drugs
Going from a low to a high elevation
Exercise with excessive perspiration
Hormone secretion
Newborn infant
Lowered oxygen tension
Stress polycythemia

Pathologic:

Acidosis
Anoxemia
Apoplexy
Atherosclerosis
Asthma
Bronchitis
Carbon monoxide poisoning
Carcinoma of the esophagus
Cholera
Chronic heart disease with cyanosis
Congenital heart disease
Diarrhea
Emphysema
Hemoconcentration leading to shock
Nephritis, rare
Perspiration, abnormal
Pneumothorax
Poisons, some bacterial and chemical
Polycythemia vera
Pulmonary disease, chronic fibrotic
Splenic tuberculosis
Splenomegaly
Suffocation
Trichinosis at times
Ulcer of pylorus with vomiting
Vomiting
X-ray exposure in moderate doses at times

Some conditions with low red blood cell counts

Physiologic:

Temporarily following injections of fluids

Pathologic:

All anemias and conditions accompanied by anemia, in varying degrees

HEMOGLOBIN CHANGES

Physiologic increase:

Changing from a low to a high altitude
At birth—after the fourth day it drops to 58-75% for the first year and then rises until it is normal at the fifteenth year

Physiologic decrease:

Temporarily after intravenous injections of large amounts of fluid
Premature children as low as 52% during the fifth month of life

Pathologic conditions with normal hemoglobin:

Often in agranulocytosis
Measles
German measles
Parotitis epidemica
Pseudoanemia Sahli
Varicella

Pathologic conditions with increased hemoglobin:

Chronic carbon monoxide poisoning and other conditions with hemoconcentration
Cardiac disease
Dehydrated states
Emphysema
Advanced empyema
Hemoconcentration leading to shock

Pathologic conditions with decreased hemoglobin:

Aleukemia
Amebiasis
Amyloid nephrosis
All anemias
Aplastic anemia
Arthritis
Banti's syndrome
Benzol poisoning

Chagas' disease
Chloroleukemia
Chlorosis
Clonorchis sinensis infestation
Coccidioidal granuloma
Diphyllobothrium latum infestation
Ectopic pregnancy
Egyptian anemia
Enterobiasis (oxyuriasis), untreated
Erythroblastic anemia
Fasciola hepatica—massive infestation
Gaucher's disease
Goat's milk anemia
Hansen's disease
Hematoxic anemias
Hemolytic anemias
Acute hemorrhage
Hodgkin's disease
Hookworm disease
Idiopathic hypochromic anemia
Progressive hypocythemia
von Jaksch's disease
Lead poisoning
Leishmaniasis (kala-azar)
Leukemia
Acute leukemia of children
Chronic leukemia of children
Malaria
Niemann-Pick disease
Ovarian abscess
Paratyphoid
Pernicious anemia
Acute febrile pleochromic anemia of Moschcowitz
Pneumonia, at times slight increase
Poisoning with acetanilid, acetophenetidin, other aniline derivatives, purine, and potassium chlorate
Pseudochlorosis infantum
Pyelitis, chronic
Raillietinosis
Scarlet fever
Schistosoma haematobium infestation
Scurvy
Sickle cell anemia and other constitutional anemias
Sprue
Syphilis and anemia
Syphilis, congenital
Taenia saginata infestation
Trichinosis
Tumors, malignant
Trypanosomiasis
Typhus
Ulcerative colitis

WHITE BLOOD CELL CHANGES

Some conditions with high white cell counts

Physiologic:
Afternoon counts are higher than morning counts
Birth
Digestion
Exercise, including crying of infants
Menstruation
Pregnancy
Pathologic:
Most acute bacterial infections, including those with abscess formation, almost anywhere in the body, one exception being the normal leukocyte count that usually attends brain abscesses
Trauma of all kinds including shock, hemorrhage, surgery, bone fractures, and severe burns
Exposure to drugs and chemicals: Adrenalin, mercury bichloride, venoms of animals such as insect bites, black widow spider bites, and snake bites
Metabolic diseases such as diabetic coma, the nephritides, and scurvy
Death of tissue such as occurs in infarction of organs including coronary occlusion or thrombosis, and embolism of any organ of the body, including the extremities
Mesenteric thrombosis is characteristically associated with a marked hyperleukocytosis
Bleeding into organs or body spaces, such as hemorrhage into ovarian cysts, hemorrhage into the kidneys, or hemorrhage into the pleural or peritoneal cavity
Malignant tumors with marked degenerative changes
Any condition in which intravascular hemolysis occurs, such as severe transfusion reactions due to incompatibility of blood, crises in sickle cell anemia, and fatal blackwater fever
Protozoal diseases during attacks, such as malaria during the chill and congenital syphilis
Parasitic infestation
Some blood dyscrasias, such as polycythemia vera and leukemias

Some conditions with low white cell counts

Physiologic:
Injections of fluids
Neurasthenia
Pathologic:
Anaphylactic shock
Avitaminosis at times
Banti's syndrome
Basedow's disease
Beriberi
Drugs, such as benzol, arsenic, lead at times, mercury, sulfonamides, aminopyrine, urethane, nitrogen mustards, 6-mercaptopurine, gold salts, chloramphenicol, and thiouracils
Empyema when many leukocytes have poured into the area
Felty's syndrome
Food allergies
Gaucher's disease
Granulomas at times, sarcoid
Infections, bacterial, such as typhoid fever and some cases of brucellosis (paratyphoid usually accompanied by a normal or slightly elevated white blood count)
Infections, viral, such as influenza, dengue, infectious mononucleosis (questionable virus), measles, pappataci fever, epidemic parotitis, poliomyelitis, vaccination, smallpox, scrub typhus or tsutsugamushi fever, and infectious hepatitis
Infestation, protozoal, such as disseminated histoplasmosis, kala-azar, malaria, trypanosomiasis, and yellow fever

Overwhelming infection, especially of the debilitated or of elderly people; may be due to bacterial organisms of an acute type or may be associated with widespread disease such as miliary disseminated tuberculosis
Hematologic disorders, such as agranulocytosis, aleukemic leukemia, aplastic anemia, infectious mononucleosis, and pernicious anemia
Irradiation, either in the form of products of fission, such as the atomic bomb or radioactive isotopes, or exposure to x-ray
Myxedema
Osteoscleroses, diffuse
Paroxysmal hemoglobinuria during attacks
Pellagra
Splenomegalic diseases other than those listed here manifesting hypersplenism
Primary splenic neutropenia
Sprue
Vaccination, occasionally
Yellow fever at times

SUMMARY OF CHANGES IN BLOOD PLATELETS

Physiologic increase:
At birth (not constant)
Marathon runners
After administration of certain medicaments, e.g., diphtheria antitoxin, epinephrine, pyrodin, and hypotonic saline and sodium nucleinate by mouth
After operations (not constant)
Blood which is shut off from circulation in a doubly ligated vein
Pathologic increase:
Bubonic plague
Often in chronic hemolytic icterus
Febrile period of acute infections
Hemolytic anemias
Chronic myeloid disease
Pneumonia
Polycythemia vera
Posthemorrhagic anemia
Sickle cell anemia
Typhus
Physiologic decrease:
After eating
After administration of certain drugs, e.g., arsphenamine, calcium, atropine, saponin, antimony, corpus luteum extract, tuberculin, and phenylhydrazine
Menstruation
Pregnancy
Senility
Pathologic decrease:
Aleukemic leukemia
Idiopathic aplastic anemia
Benzol poisoning
Acute infections during incubation periods
Jaundice
Kala-azar
Acute leukemia
Diseases of the liver
Lymphocytic leukemia
Panmyelophthisis
Pernicious anemia
Purpura, thrombocytopenic
Scarlet fever
Trauma
Uremia
Varicella at beginning of fever

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Section Two

BLOOD GROUPS, TRANSFUSION, AND MEDICOLEGAL APPLICATIONS*

The subject of blood grouping and transfusion, with medicolegal applications, has assumed such gigantic proportions within recent years that to cover all phases of the subject thoroughly would require not one chapter but perhaps a library of information. It is the purpose of this section to discuss the subject primarily for use in the routine examinations of patients and donors prior to and at times following transfusions; in the examination of patients and at times donors for unusual blood factors or types; to explain the use of the blood group factors and types in genetic studies for clinical and medicolegal applications; in the identification of blood stains, semen, saliva, and other body fluids; and in transfusions and other applications. In addition to detailing methods for routine examination of blood, specialized technics will be described for the so-called "rare" blood factors, the protection of the recipient of a transfusion, the protection of the donor, the liability laws involved in blood transfusions, the implication of the blood factors and their importance in pregnancy, and other allied subjects.

Thorough and strict adherence to technic is a prerequisite for all laboratory investigation, but in the field of blood grouping serology improper or sloppy methods or failure to use enough controls and to use them properly can result in loss of life. The salient facts regarding technics of handling material for blood grouping experiments will be given in detail. Outdated or weak-reacting reagents contribute to mistakes in the serologic laboratory. This subject and the "blind"[1] test as a means of preventing or correcting errors will be included. The latest advances in methods will be given, any obsolete method will be deleted, and there will be a comprehensive review of all blood factors known to date, together with synonyms when such exist. An attempt will be made to simplify a complicated subject, but oversimplification will be avoided inasmuch as a false sense of security in a knowledge not possessed might lead to death of a patient.

In accordance with the recommendations of the Committee on Nomenclature of the American Medical Association, blood agglutinogens will be designated by ordinary type, genes by *italics,* and blood factors by **bold-faced type.** In the main, the nomenclature suggested by A. S. Wiener will be followed. The English nomenclature will be discussed but will not be used in the text except for discussion and comparison with the International Nomenclature.

Since the first edition of this textbook, which was published in 1935, blood grouping has developed into a science of vast proportions. Many of the old concepts have had to be modified, and as new blood factors have been discovered even those findings assumed to have been correct but a few short years ago have had to be modified in the light of modern investigation. Although much of the knowledge concerning blood groups and blood factors is primarily useful in the field of legal medicine and genetics, much applies to clinical practice. The serologist and technologist working in blood banks or in clinical laboratories today are required to have a working acquaintance with all the developments in this complex subject. Many of the older methods have given way to new and improved technics, and these must be applied skillfully so as to avoid errors.

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There are at the present more than 50 known factors that determine individual serologic differences among human beings. Of these, 3 are of paramount importance, **A**, **B**, and **Rh**$_0$, since these are the most frequent sources of clinical problems. Although opportunity for isosensitization, by pregnancy and transfusion, to the other blood factors, such as **M**, **N**, **S**, **U**, **P**, **rh′**, **rh″**, **hr′**, **hr″**, **K**, **Duffy**, etc., constantly occur, examples of isosensitization are relatively uncommon, indicating that these are less antigenic than blood factors **A**, **B**, and **Rh**$_0$. Certain individuals have a special propensity to form antibodies, as shown by reports of instances of multiple sensitization to a number of different blood factors more often than can be ascribed to chance alone. In some instances the sensitized individuals have autoantibodies as well as group-specific antibody; for example, Wiener et al.[2] have not infrequently found autoantibodies in the serum of individuals sensitized to both factors **hr′** and **hr″**.

Individuals who are negative for the **Vel**, **Tj**^{a}, **Rh**^{A}, **Rh**^{B}, **Rh**^{C}, **Rh**^{D}, **hr**^{S}, **U**, and **I** factors are quite rare, so that opportunities for sensitization are few and seldom occur. If incompatibilities are detected in cross-matching tests preliminary to a transfusion, the problem of finding a compatible donor will be simplified if the blood factor or factors responsible for the incompatibility can be identified. One way of finding such a donor is to test the patient's serum against a panel of selected group O cells known to contain a varied assortment of the numerous blood factors that have been identified to date, as suggested by Unger in 1954.[3] By the use of such a panel, Wiener et al. quickly identified an antibody in the serum of a mother of an erythroblastotic baby as anti-**S**. The limitation of such panels is that they are unlikely to include bloods of the very rare types and are therefore of no value for identifying antibodies such as anti-**U**, anti-**I**, anti-**Vel**, anti-**Kj**^{a}, etc.

The most important clue to identifying antibodies is that antibodies for blood factors of different blood group systems behave differently. Rh-Hr antibodies can generally be recognized by the sharp reaction given by most of the known technics: conglutination, antiglobulin, and especially the proteolytic enzyme method. Cells that are maximally coated with any antibody of the Rh-Hr system generally give equal titers with the same antiglobulin serum. **Duffy** and **Kidd** antibodies, however, generally act best by the antiglobulin method, and cells maximally coated with such antibodies generally react with antiglobulin sera to $\frac{1}{10}$ the titer of cells maximally coated with Rh antibodies. **Kidd** antibodies react best in serum from freshly drawn blood. **K-k** antibodies have properties intermediate between those of the **Rh-Hr** and the **Duffy** antibodies. Antibodies of the M-N system not infrequently react on saline-suspended unmodified cells, but when the cells are treated with proteolytic enzyme, the type-specific reaction disappears and is replaced by a nonspecific one. **Vel** antibodies can be recognized by their capacity to produce hemolysis in vitro as well as from the very low incidence of compatible red blood cells.

Caution

With the increase in the number of transfusions and the discovery of so many blood factors not heretofore known, it has become increasingly evident that blood grouping is a highly specialized field, and these delicate tests must be carried out with precision by specially trained personnel if fatal hemolytic reactions are to be avoided.

Chapter 72

General considerations

ADDINE G. ERSKINE

HISTORY OF BLOOD TRANSFUSIONS

The first record of a blood transfusion is referred to in classical literature: Medea attempted to rejuvenate old Anchises by removing blood from his cervical vessels and replacing it with juvenile blood (Ovid). Obviously no genuine blood transfusion could have been performed prior to the discovery of the circulation of blood. Clark and Henshaw, soon after the discovery of the circulation of blood by William Harvey in 1616, began to make animal experiments on blood transfusion. Denys and Emmerez of Paris in 1667 made a direct transfusion of the blood from the carotid artery of a sheep into the cubital vein of a human patient, and good results followed. Others repeated this, but disastrous results occurred in some cases, so that it was abandoned.

Blood transfusion was revived during the past century. However, because severe reactions and death followed the transfusion of incompatible bloods, the procedure was practically abandoned for many years. It was revived only when methods were discovered for determining the compatibility of human bloods. At the present time methods of blood grouping and blood matching have eliminated many of the dangers incident to transfusion. It is now known that in most instances the unfavorable reactions are due either to hemolysis or to agglutination of either the donor's or the recipient's blood cells or to both.

HISTORY OF DISCOVERY OF THE FOUR PRINCIPAL BLOOD GROUPS—O, A, B, AND AB

In 1901 Landsteiner[4] established the basis for the present methods of determining compatibility or incompatibility of bloods for transfusions. He discovered that all human beings can be divided into 3 distinct groups. He showed that the identity of each group depended upon the presence on the red blood cells of "isoagglutinogens." The 3 blood groups he called A, B, and C (not O). Group C was later called O. A fourth group was discovered by von Decastello and Sturli,[5] the latter a pupil of Landsteiner's. This was the AB group. It was found that in the case of group A, the serum reacted upon the corpuscles of group B, causing them to clump or agglutinate, but the serum of group A did not react upon the corpuscles of group A. The corpuscles of group A, in turn, were agglutinated or reacted upon by the serum of group B, but the serum of group B did not react upon the corpuscles of group B. It is obvious, therefore, that normally the corpuscles are apparently not sensitive to their own sera or generally not to sera of other individuals belonging to the same blood group (see subgroups, p. 1470).

Four principal blood groups are now recognized, designated as groups A, B, AB, and O. These belong to a blood group system designated the A-B-O system. This is the International Classification of the blood groups. The 2 other classifications of the same blood groups, the Moss[6] and Jansky,[7] are no longer used, but merely as a matter of historical record their equivalents according to the International Classification are given in Table 296.

Approximately 45% of American and European peoples belong to group O, 42% to group A, 10% to group B, and 3% to group AB. The International Nomenclature, officially recognized by the Health Committee of the League of Nations, is the same as the original classification of Landsteiner and von Dungern and Hirszfeld, and it is the nomenclature of choice. To avoid confusion, the group names of Moss and Jansky have been eliminated from medical literature.

A further interesting communication upon blood groups was that by von Dungern and Hirszfeld,[8] who discovered the existence of subdivisions or subgroups in 2 of the 4 human blood groups. These subdivisions have now been extended to include many more.

The substances that characterize the A-B-O blood groups are present in practically every tissue of the body and have been found in soluble form in the serum, saliva, gastric juice, semen, urine, etc. of the so-called "secretors."

DISCOVERY OF OTHER BLOOD TYPES AND BLOOD FACTORS AND CLASSIFICATION INTO SYSTEMS

In 1927 Landsteiner and Levine[9, 10] noted that when certain immune sera from rabbits previously injected with human blood were absorbed with certain samples of human blood, they still contained agglutinins acting on the majority of bloods of all 4 groups but not upon all. This led to the discovery of the blood factors **M** and **N**, which determine the agglutinogens M and N. Either M or N agglutinogens or both were found to be present on all human blood cells, but natural anti-**M** or anti-**N** agglutinins are only rarely found in human sera. Such antibodies are produced by injecting rabbits with the appropriate type of cells. Research since the initial discovery has demonstrated that all human bloods may be divided into 3 types, M, N, and MN. Agglutinogens M and N are transmitted by heredity just as are the agglutinogens of the A-B-O system.

The **M** and **N** factors are of almost no importance with respect to blood transfusion work because there are hardly ever any anti-**M** or anti-**N** agglutinins in normal human blood sera, and M and N agglutinogens are apparently only feebly antigenic for human beings. (For exceptions refer to p. 1552.) They are important in questions of paternity, in family studies, and in other medicolegal work but are unreliable in the identification of dried blood stains. This is described on pp. 1599-1618ff.

The discovery of the Rh factor by Landsteiner and Wiener in 1937 and the report of its finding in 1940[11] constitute the most important advance in the field of blood grouping serology since the discovery of the 4 principal blood groups. With its discovery blood grouping serology was given a new impetus. New terms are now used to describe attributes of the red cell antigens, the nature of antibodies has been studied and further clarified, and the study of the genetics of the blood types has been amplified.

Blood group systems: The various blood groups now are recognized as belonging to certain systems, of which there are 9 principal ones, and some unclassified. These 9 are A-B-O (and Lewis), M-N-S-U, P, Rh-Hr, K-k (Kell), F-f (Duffy), J-j (Kidd), Lu (Lutheran), and Di (Diego). The most complex of all the systems is the Rh-Hr.

Other important discoveries: Inheritance of the 4 Landsteiner blood groups was demonstrated by Ottenberg and Epstein in 1908[12] and von Dungern and Hirszfeld in 1910.[13] The exact mechanism of heredity of the blood groups was solved by Bernstein in 1925.[14] The inheritance of the Rh-Hr types was worked out by A. S. Wiener and his collaborators. Other important discoveries have been reported, and these will be listed when the individual systems are discussed.

GENERAL TERMINOLOGY USED IN BLOOD GROUPING SEROLOGY*

Antigen: The term "antigen" refers to any substance which, if injected into human beings or other animals, stimulates the production of antibodies, immune bodies capable of reacting with or destroying the antigen. In the case of blood grouping, the antigen is found on the red cells and is also recoverable in solution in certain body fluids and secretions in individuals known as secretors.

Antibody: An antibody is an immune substance formed in response to the stimulus of an antigen. It reacts with the antigen that called it into being. It is found in the plasma and is recoverable in the serum or plasma. Cross reactions also occur; for example, anti-**A** can react with sheep cells as well as with human A and AB cells.[15]

Agglutinogen: An agglutinogen is a substance present on the surface of red blood cells which, when combined with the corresponding specific antibody, results in clumping or agglutination of the red cells. An agglutinogen may have many factors, each factor capable of stimulating production of or combining with a specific antibody.

Blood factor: Agglutinogens of red cells are generally characterized by a number of "blood factors," which are serologic specificities by which agglutinogens are recognized. An agglutinogen may have multiple factors, all transmitted as a block by heredity. The blood factors are not necessarily limited to the cells of human beings. For example, if a blood contains factor **A**, the cells are clumped by a serum that contains **anti-A** agglutinin, but this does not necessarily mean that those blood cells have agglutinogen A of the **human** group A blood. There are many agglutinogens other than that of the human blood group A that react with particular anti-**A** sera, specifically an agglutinogen of sheep cells. As another example, some of the blood factors **B** which characterize agglutinogen B of human blood are shared by agglutinogens of the red cells of rabbits and other rodents.[16]

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*For a more complete glossary, refer to pp. 1487ff.

As another example of blood factors present in a single agglutinogen, blood of type Rh$_1$ may contain, in addition to factors **Rh**$_0$ and **rh′**, many other factors such as **rh**$_i$, **Rh**^{A}, **Rh**^{B}, **Rh**^{C}, **Rh**^{D}, **rh**^{G}, **hr″**, **hr**^{S}, and **Hr**, but it lacks **rh″**, **hr′**, **hr**, **rh**^{w}, **rh**^{x}, **rh**$^{w}_{2}$, and **hr**^{V}. Wiener predicted that the number of blood factors characterizing any Rh-Hr agglutinogen is theoretically unlimited and depends largely on one's enterprise and ingenuity in finding antisera of new specificities, but he made no attempt to anticipate the nature of the specificity of blood factors still to be found since this is generally unpredictable. New blood factors usually are identified after an antibody has been found in a serum following transfusion or in pregnancy.

The prefix "iso-": The prefix "iso-" in blood grouping serology usually refers to human agglutinogens and agglutinins in the A-B-O system, except when it is used to describe a reaction of isoimmunization. In the latter case it means formation of antibodies by a member of a species of an animal or by a human being in response to an antigen present on the cells of a member of the same species. For example, a human being of type rh (Rh negative) may form anti-Rh isoantibodies as a result of a transfusion of human blood containing the Rh agglutinogen; the transfused person has been isoimmunized against the Rh agglutinogen. Such isoimmunization occurs in pregnancies as well as in transfusions (pp. 1508-1509).

Agglutinin: The term "agglutinin" refers to an antibody in the blood serum which has the ability of reacting with its specific agglutinogen to cause the cells containing that agglutinogen to clump or "agglutinate." Refer to p. 1512 for the nature of antibodies.

Agglutination: Agglutination means the clumping of cells. In blood group nomenclature, agglutination refers to the clumping of red cells by action of a specific serum. False appearance of agglutination due to causes other than antigen-antibody reactions is referred to as **pseudoagglutination.** An example of pseudoagglutination is rouleaux formation, such as is found especially in individuals with very high sedimentation rates.

Blood group system: The division of various blood types into systems depends on their hereditary transmission, and knowledge of such transmission must often depend on family studies along with serologic investigations. Certain terms must be used in these studies.

Genotype: Genotype refers to the constitutional make-up of an individual as determined by heredity. Genotypes are transmitted through the germ cells by means of their chromosomes. The make-up of an individual in terms of his genes is known as his genotype.

Gene: Genes are units of inheritance. These genes are arranged in a linear fashion along the chromosomes.

Phenotype: Phenotype refers to the characteristics of individuals of a species as determined by direct observation, measurement, or chemical or serologic testing.

AT WHAT AGE DO AGGLUTINOGENS AND AGGLUTININS APPEAR IN THE BLOOD?

Kemp[17] showed that the isoagglutinogen could first be demonstrated in the red blood cells of a fetus of 37 days and demonstrated that the sensitivity of the cells increases throughout antenatal and postnatal life up to the age of 20 yr., after which time it remains constant.[18] He also noted that the red blood cells of the newborn infant generally have only 20% of the sensitivity to agglutination that adult blood cells have. Expressed in other terms, a serum with a titer of 500 against adult corpuscles would usually agglutinate the corpuscles of the newborn infant only up to a titer of 100. Thomsen and Kettel[19] determined the titer of the isoagglutinins in the sera of individuals of various ages and found that isoagglutinins begin to appear in the latter half of antenatal life and rapidly increase in titer after birth up to the age of puberty, when the titer gradually diminishes. The average titer of the anti-**A** agglutinin is generally higher than that of anti-**B.** Wiener states that whereas isoagglutinogens are demonstrable at birth, only about half of all newborn infants have demonstrable isoagglutinins. Hirszfeld[20, 21] believed that any isoagglutinins at birth are derived from the mother by filtration through the placenta. (See factor **C** of the A-B-O system, p. 1472.) Smith[22] studied the isoagglutinogen and isoagglutinin of the blood of infants from day to day during the first few weeks of postnatal life and found that whatever isoagglutinins were present at birth diminished in titer or disappeared during the first 10 days of life, after which time new isoagglutinins appeared.

Bornstein and Israel[23, 24] made a study of agglutinogens in fetal erythrocytes, particularly the M and N agglutinogens. Moureau[25] demonstrated the **M** and **N** factors in very young fetuses. Hyman[26] found **M** and **N** demonstrable in 3 fetuses but failed to obtain either **M** or **N** in the fourth, at 3 mo., the cells of which exhibited the isoagglutinogen B. She left the question open whether they had not yet developed at that time. Bornstein and Israel's study[23] comprised a larger series of fetuses to determine whether or not their agglutinogens are detectable with sera and testing fluids used in transfusion work and in legal tests. By including the Rh factor in the investigation, information could be obtained on the value of tests performed with fetal blood for investigations of isoimmunization as a cause of habitual abortion, according to Levine et al.[27] Bornstein and Israel's series comprised 19 premature fetuses, only those showing gross evidence of maceration being omitted. Blood was taken from the heart, suspended in physiologic saline solution, and examined the same day with the centrifuge method. Examination of parents was possible in some cases. Various human **A** and **B** antisera and **M** and **N** testing fluids were used, which gave positive agglutinations with the corresponding cells in a dilution of 1:8. For the Rh tests the test tubes were incubated for 1 hr. at 37° C. before centrifugation.

The fetuses varied in length from 7 to 50 cm., the average being 28.6 cm. The distribution of agglutinogens was independent of the length of the fetuses and did not grossly deviate from the percentages in the population. No fetus without either **M** or **N** was encountered, and no combination parent type M, fetus N, or vice versa, occurred. The Rh factor was demonstrable in 4 out of 5 fetuses. This confirms the indirect evidence that the Rh factor is present early in fetal life.

Table 292 gives the summary of Bornstein and Israel's work.

Table 292. *Blood groups and blood types of fetuses and parents (presence or absence of Rh factor tested in Nos. 15-19 only)*

Fetus				
No.	*Length (cm.)*	*Agglutinogens*	*Mother*	*Father*
1	21	O MN	O M	
2	7	O MN	B M	
3	13	A M	A M	
4	47	O MN		
5	23	A M		
6	13	O M	O MN	O MN
7	30	A MN	A MN	A MN
8	50	A M		
9	42	A N		
10	27	A MN		
11	19	O N		
12	26	B M		
13	30	A N		
14	33	B N	A MN	
15	41	AB M Rh+	A MN Rh+	
16	42	A M Rh+	O MN Rh+	
17	39	A MN Rh−	A MN Rh−	
18	17	O N Rh+	A N Rh+	
19	21	B MN Rh+	A MN Rh−	B N Rh+

This research demonstrated agglutination of A, B, M, N, and Rh in the heart's blood of 19 fetuses. This seems to prove that agglutinogens are actually developed long before birth and in sufficient strength to make the examinations reliable enough for scientific purposes.

IDENTIFICATION OF BLOOD GROUPS

Blood groups are identified by means of agglutination reactions—the clumping of cells by action of a serum. The antigen or agglutinogens are on the blood cell surfaces, which makes them agglutinable or able to clump when mixed with a serum containing the corresponding antibody or agglutinin. In the A-B-O system the agglutinogens are classified as A or B and the agglutinins as anti-**A** or anti-**B**. (The so-called "O" agglutinogen will be discussed elsewhere, p. 1454). Each agglutinin in the serum acts upon cells containing the corresponding agglutinogens; thus anti-**A** clumps cells that contain agglutinogen A, and anti-**B** clumps cells that contain agglutinogen B. Group O has neither A nor B agglutinogen, group A has A agglutinogen, group B has B agglutinogen, and group AB has both A and B agglutinogens.

Antibodies in the sera are designated as either anti-**A** or anti-**B**. Group O has both anti-**A** and anti-**B**, group A has anti-**B**, group B has anti-**A**, and group AB has neither antibody. Anti-**A** will cause clumping of cells containing agglutinogen A, and anti-**B** will cause clumping of cells containing agglutinogen B. An anti-**A** serum thus will cause clumping of cells of groups A and AB, whereas an anti-**B** serum will cause clumping of cells of groups B and AB. Group O cells will not be agglutinated by either serum.

All blood groups and types are identified by their agglutinogen content; thus type M has agglutinogen M, type N has agglutinogen N, and type MN has both agglutinogens M and N. Type Rh_0 has agglutinogen Rh_0, etc.

The serum that acts upon cells containing agglutinogen M is called anti-**M**, and the serum that acts on cells containing agglutinogen N is called anti-**N**. This nomenclature is used in all the systems. Anti-**M** and anti-**N** agglutinins are not found naturally in human serum except in extremely rare cases. Both anti-**A** and anti-**B** are natural antibodies. Further identification of blood types by their factors is found throughout this section.

COMPOSITION OF A-B-O SYSTEM

Whereas group O cells have neither A nor B agglutinogen, the serum contains both anti-**A** and anti-**B** agglutinins. The cells of group O, therefore, are not agglutinable by any sera of this system, but the serum of group O will cause agglutination of cells of groups A (containing A agglutinogen), group B (containing B agglutinogen), and group AB (containing both A and B agglutinogens).

In group A the cells have agglutinogen A and the serum contains agglutinin anti-**B**. Thus group A cells are agglutinated by group O serum (containing anti-**A**) and by group B serum (containing anti-**A**). The serum of group A, which contains anti-**B** agglutinin, will cause clumping of cells of group B (containing B agglutinogen) and group AB (containing A and B agglutinogens).

In group B the cells have agglutinogen B and the serum contains agglutinin anti-**A**. Group B cells therefore are agglutinated by group O serum (containing anti-**B**) and group A serum (containing anti-**B**). The serum of group B contains anti-**A** agglutinin and therefore causes clumping of cells of group A (containing A agglutinogen) and group AB (containing A agglutinogen as well as B agglutinogen).

Group AB blood has both agglutinogens A and B on the cells and therefore these cells are agglutinated by group O serum (containing anti-**A** and anti-**B**), group A serum (containing anti-**B**), and group B serum (containing anti-**A**). The serum of group AB, however, has neither anti-**A** nor anti-**B** agglutinin and therefore will not cause clumping of cells of any of the four groups.

No reaction occurs when either the agglutinogen or the agglutinin is lacking or when the agglutinin and agglutinogen are not specific for each other, that is, **not** designated by the same letter.

Anti-**A** is sometimes called alpha (α), and anti-**B**, beta (β). Anti-**A** reacts only with cells containing A agglutinogen, and anti-**B** reacts only with cells containing B agglutinogen.

It is now generally known that group O cells are characterized not only by absence of agglutinogens A and B but also by the presence of a special agglutinogen O. Only rarely do natural antibodies for agglutinogen O occur in human sera, and these are found in subgroups A$_1$B and A$_1$, in animal sera, especially from cattle and eels, and they have been produced by heterogenetic immunization of goats with Shiga bacilli. These antisera are of low titer and low avidity and give a marked gene dose effect, so that bloods containing the property in a single dose, for which such tests would be most useful, generally give indistinct reactions.

Table 293. *Composition of A-B-O blood groups*

Blood group	*Agglutinogen on cells*	*Agglutinin in serum*	*Cells are agglutinated by sera of group*	*Serum causes agglutination of cells of group*
O	Neither A nor B	Anti-**A** and Anti-**B**	None	A, B, AB
A	A	Anti-**B**	O, B	B, AB
B	B	Anti-**A**	O, A	A, AB
AB	A and B	None	O, A, B	None

Table 294. *Reactions of serum of A-B-O groups with cells*

Serum of group	*Reaction on red blood cells of groups*			
	O	*A*	*B*	*AB*
O	–	+	+	+
A	–	–	+	+
B	–	+	–	+
AB	–	–	–	–

Table 295. *Reactions of cells of A-B-O groups with serum*

Cells of group	*Reaction with serum of groups*			
	O	*A*	*B*	*AB*
O	–	–	–	–
A	+		+	–
B	+	+	–	–
AB	+	+	+	–

Table 296. *Comparison of blood group nomenclature in A-B-O system*

International or Landsteiner classification	*Jansky*	*Moss*	*Approximate % of Americans (Caucasoids) in each group*
O	I	IV	45
A	II	II	42
B	III	III	10
AB	IV	I	3

Extracts of *Ulex europaeus* seeds give agglutination reactions resembling those of anti-**O** serum, but they are not specific for blood group O, since the *Ulex* seed extract, like anti-**O** sera in general, tends to react most strongly not only with red cells from individuals of group O but also of subgroup A$_2$. The reagent is now designated "anti-**H**" and is most useful in diagnosing the secretor types. The reagent gives sharp reactions despite its low titer and is used for testing saliva of individuals of any A-B-O group.[28, 29] The secretor tests are described on pp. 1542-1544.

SAFE TRANSFUSIONS

In order to ensure safe blood transfusions (pp. 1464ff) that is, in order to prevent what is known as incompatibility reactions, certain preliminary examinations must be made of both the recipient's and the donor's blood. These are as follows (details in Chapter 73):

1. Obtain the blood group of both the recipient and the donor by testing the cells with known anti-**A** and anti-**B** sera, and check these results by testing each of the blood sera

(recipient and all donors), previously inactivated, against known A_1 and B cells.

2. Obtain the subgroup if the recipient is group A or group AB.
3. Cross match the recipient's serum with the donor's cells and the recipient's cells with the donor's serum.
4. Determine the Rh factor in the cells of the recipient and of the donor.
5. During the cross-matching tests, make tests also on both the donor's and the recipient's sera for possible Rh-Hr or other antibodies; such tests are called "sensitivity tests." Refer to p. 1482.

Caution: A serologic test for syphilis should be performed on all prospective blood donors to exclude syphilis. It is well to bear in mind the possibility of transmitting malaria in blood transfusions. It is best not to transfuse blood of an allergic individual. The subject of transference of homologous serum jaundice is discussed on pp. 1568, 1585, and 1591.

SPECIFIC AGGLUTINATION

When the red blood cells of a normal human being are suspended in physiologic saline solution, an even suspension results, although upon standing the cells will settle. If this even suspension of cells is mixed with serum of the same individual or of another individual of the same blood group, the suspension is apparently unaffected. If, however, blood serum of an individual of an incompatible blood group is added to this even cell suspension, the red blood cells come together in clumps, which is the phenomenon of agglutination and is the basis for division of all individuals into the 4 principal groups and the means of identifying the numerous blood types and blood factors. The agglutination of cells when mixed with serum depends upon a specific reaction between 2 substances, 1 on the red cells called "agglutinogen" and 1 in the serum called "agglutinin." Each agglutinin is specific for a certain agglutinogen and each agglutinogen reacts with a specific agglutinin, although in the light of modern investigation it is incorrect to assume a simple 1:1 correspondence between agglutinogen and antibody, as Wiener and others have pointed out from time to time. For example, in the Rh-Hr system, each agglutinogen is characterized by multiple serologic factors which are inherited in a block by means of corresponding Rh-Hr allelic genes.

Agglutination tests may be made either on the open slide or in tubes. In either case cells are mixed with serum. Either the cells or the serum may be unknown, or if both are known, this is called a "control." If the cells are unknown, the agglutinin in the serum must be known in order to identify the cells. If the agglutinin is unknown, then known cells must be used to identify the serum. Suitable controls must always be made with every blood grouping experiment to ensure reliable results.

True agglutination occurs when definite clumps of red blood cells are found. When the concentration of antibodies in the serum is high (high titer serum), the clumps will be large, brick red, and definitely separated from each other, or one large clump may appear, and individual cells will not be identifiable in the clumps upon microscopic examination. When high titer serum is used, macroscopic examination reveals the agglutination in most cases.

There are many degrees of agglutination, designated by Unger as +++S, +++W, ++S, ++W, +S, +W, ±, and negative (−). S = strong; W = weak. The weaker agglutination reactions are visible only under the microscope, and the color of the clumps is not as red as when the clumps are large; often the clumps appear star-shaped and brownish. One must guard against false appearance of clumping, settling of cells, and other nonspecific agglutination reactions. By adding a drop of saline to the slide after sufficient time has been allowed for agglutination to take place, then rapidly rotating the slide and reading microscopically, such false appearance of agglutination and settling of the cells will usually be avoided.

False negative reactions

If testing sera are of high titer, the reactions are so definite with the open slide method that it is practically impossible to mistake them. However, if one uses whole blood as the unknown in testing for A-B-O blood groups or in the controls, the reactions are difficult to read if the mixture of blood and serum is not spread thin on the glass slide or if saline is not added after sufficient time has elapsed for the reaction to take place. When testing unknown sera against known cells, the antibody concentration is not controlled, and weak positive reactions might occur. The same is true in cross matching, in which the drops must be searched diligently for slight clumping. Likewise, antibody titer gradually decreases in ampuled sera, so that controls must always be made before using such testing sera.

If weak-reacting blood grouping sera are used, either the reaction might be overlooked or it may fail to take place. If a weak anti-**A** serum is used, it could fail to detect the weak A agglutinogen of subgroup A_2 or A_2B cells. Thus a subgroup A_2 blood would be erroneously classified as group O, and a subgroup A_2B would be erroneously classified as group B. If the anti-**B** serum is weak, it might fail to detect the B agglutinogen on group B and AB cells, erroneously classifying a group B blood as group O and a group AB blood as group A. In all blood grouping in the A-B-O system, tests must be made using the serum of the unknown against known A and B blood cells, as well as tests on the cells using known antisera. This will help eliminate errors which may be made inadvertently, especially in the subgroups (refer to subgroup B_3, p. 1474).

Another source of error may be low sensitivity of agglutinogens, which is seen when testing blood of newborn infants or at times when using preserved blood suspensions. As already mentioned, one of the most common errors is to mistake subgroup A_2B blood for group B because of the weak reactivity of the A agglutinogen in such blood. The use of high-titered anti-**A** sera will obviate such results. (See p. 1459.)

NONSPECIFIC AGGLUTINATION

False positive reactions

Pseudoagglutination: Pseudoagglutination is the false appearance of agglutination that is not true agglutination at all but usually rouleaux formation of the red blood cells. It simulates true agglutination. It is produced by high concentration of serum and high temperatures and has been noted when tests are made on open slides in an air current. It occurs in the slide test because of the low dilution of the serum and because the large surface favors evaporation and concentration of the serum. Serum from patients with rapid sedimentation rates will produce rouleaux formation with any red blood cells, including their own.

One must be cautious to avoid pseudoagglutination when using gum acacia, gum tragacanth, and gelatin. Care must be exerted in reading reactions when trypsinated or ficinated cells are used. A negative control for comparison, prepared just like the test, must always accompany such tests.

The difference between pseudoagglutination and true agglutination can be readily seen under a microscope. In rouleaux formation the red blood cells are arranged in piles like a stack of coins. By adding a few drops of saline, rouleaux formation or pseudoagglutination will usually disappear, whereas true agglutination either is not affected by this procedure or at times is made even more apparent.

Bacteriogenic agglutination — Huebner-Thomsen or panagglutination phenomenon: Bacteriogenic agglutination is a phenomenon unrelated to isoagglutination and results from bacterial action. It does not occur when fresh blood and serum are used. It is seen in some samples of stored blood in which the cells are clumped by any normal human serum, including that of the individual from whom the blood was derived and also by group AB serum which ordinarily does not clump any cells.

Bacterial contamination also affects blood sera which then cause agglutination of red cells of several groups as well as their own groups. It may be avoided by using only freshly drawn or sterile blood.

Autoagglutination: Autoagglutination is a condition in which the serum agglutinates its own red blood cells, due to the presence of an absorbable agglutinin in the serum and a corresponding agglutinogen on the cells. Autoagglutinins act not only on the subject's own cells but also on the cells of all other human beings, regardless of blood group, and are therefore **panagglutinins.** The reaction occurs most often in tests made at low temperature. It usually disappears upon warming and reappears upon chilling.

In certain pathologic conditions the cold autoagglutination titer is considerably increased so that the reaction may occur also at room temperature. Such conditions are atypical pneumonia, paroxysmal hemoglobinuria, syphilitic or hypertrophic cirrhosis of the liver, hemolytic icterus, Raynaud's syndrome, trypanosomiasis of man and animals, severe anemias, and other conditions. These autoagglutinins at room temperature are a source of error in blood grouping, especially when the titer is high. In some cases of Hodgkin's disease and febrile tuberculosis it is impossible to perform a red blood count, as pointed out by Gradwohl, and also difficult to determine correct blood groups.

The serum can be freed of the interfering autoagglutinins by separating it from the cells at 0°-5° C., at which temperature the blood cells absorb the autoagglutinins. The cells should be washed with warm saline (37° C.) before cross matching.

"Cold" agglutination: Cold agglutination is due to the taking up of adsorbable antibodies in the cooled state. Since cold agglutination occurs in human bloods exposed to temperatures 0°-15° C. and is prevented by higher temperatures, this factor must be borne in mind when conducting tests.

Secondary coagulation simulating agglutination: Tests made with unwashed cell suspensions often show secondary coagulation or the presence of a clot. This does not occur with washed cell suspensions or when inactivated sera are used for the tests.

False agglutination by umbilical cord serum: Wiener observed that some cord sera produce a phenomenon resembling true agglutination. While testing a series of cord sera with A and B blood cell suspensions by the open slide method, he found that when the slide was tilted back and forth the cells came together in clumps but that these clumps broke up again if the slide was allowed to remain at rest. The process could be repeated indefinitely. It was demonstrated that Wharton's jelly from the umbilical cord was responsible for the phenomenon.

THE "BLIND" TEST

In 1952 Wiener described the advantages of using his so-called "blind" test.[28] He stated that it is often difficult to make one's readings entirely objective so that subconsciously, for example, one tends to shake the tubes a little harder when a negative reaction is anticipated. Such pitfalls can be avoided only by taking the readings blind, that is, one investigator sets up the tests while a different worker reads the reactions without knowing the exact nature of the mixture which is being examined for clumping. If the observations are really objective and not due to artifacts, they should be reproducible when the tests are made on a different day with the tubes arranged in a different order, so as to avoid the possibility of subconscious bias in the person making the readings.

CONTROLS

As in other laboratory tests, the use of controls in testing unknown blood is imperative. Even when taking such precautions as using no antisera beyond their expiration date, the blind test, and the best technic possible, mistakes can be made, and controls, when properly carried out, will prevent errors which could result in a transfusion reaction or death. These controls may be made simultaneously with the tests or they may be made just prior to testing the unknown bloods. When, because of the nature of the test, controls cannot be made, using the "blind" technic and making the tests in duplicate will help prevent mistakes.

ISOHEMOLYSIS

Certain unheated blood sera, when mixed with incompatible blood cells, will dissolve the cells instead of causing agglutination. This reaction occurs between sera of human blood and human blood cells and is not to be confused with hemolysis of certain blood cells by specific amboceptor sera. This reaction is due to isohemolysins normally present in the sera and is not caused by the isoagglutinins normally found in human sera. Thus it is not unusual for the serum of group O to hemolyze the blood cells of group A or even B or AB. The sera of group AB individuals do not contain isohemolysins. In blood grouping work, hemolysis must be considered equivalent to agglutination. Usually agglutination of the cells occurs first, followed by hemolysis. This is not necessarily true, for we have frequently observed the dissolving of erythrocytes without preliminary agglutination. In one instance a group B serum caused hemolysis of both A_1 and A_2 cells without first agglutinating them when the serum was unheated. After inactivation this same serum gave a titer of 80 units against the A_1 cells but required 10 min. to agglutinate the A_2 cells.

The drop of blood in which isohemolysis occurs can be observed in the process of clearing even by macroscopic examination. The orange-colored cloudy suspension will clear and take on a reddish cast. Observation under a microscope often will disclose some erythrocytes not hemolyzed.

Isohemolysis can be prevented by inactivating the serum at 56° C. for 30 min. to destroy the natural complement.

Isohemolysis is important because it may mask isoagglutination, and, if unnoticed, may be mistaken for a negative reaction and lead to incompatible blood transfusions. It is overcome in the usual grouping test by inactivating A and B antisera.

Chapter 73

The technic of blood grouping

ADDINE G. ERSKINE

GENERAL CONSIDERATIONS

Strict adherence to all details is mandatory in blood grouping technic. All glassware must be scrupulously clean and dry. Whenever possible, the use of expendable equipment is recommended. Soda straws are often used as a substitute for droppers. Expendable glass droppers (Fig. 468) are available from a number of manufacturers. Expendable slides are not necessary but are available if desired. When wax ring slides are used, the slides must be clean, fat-free, and dry before the rings are made. Such slides should be used only once. When the welled slides (Fig. 469) are used, they must be washed first by hand, then in running water, and then thoroughly dried. Ceramic slides (Figs. 470 and 471) are useful and may be used over and over, provided all traces of blood or blood products are removed from the slides. These, too, should be washed in running tap water and then dried before use. It is best to have a large container of water at hand into which the slides may be placed until ready to be washed.

Droppers should be soaked in water and then washed in running water to make sure that all blood products have been removed, if they are to be used again for blood grouping tests. Pipets must be thoroughly rinsed and then washed, preferably in a pipet washing device. If no such device is available, the pipets should be allowed to soak in a liter cylinder, then rinsed with distilled water, and dried in an oven.

All antisera must be stored in a refrigerator at 2°-5° C., when not in use. They must not be permitted to stand at room temperature unless needed at that time. Never use outdated testing reagents.

Blood to be tested may be kept refrigerated

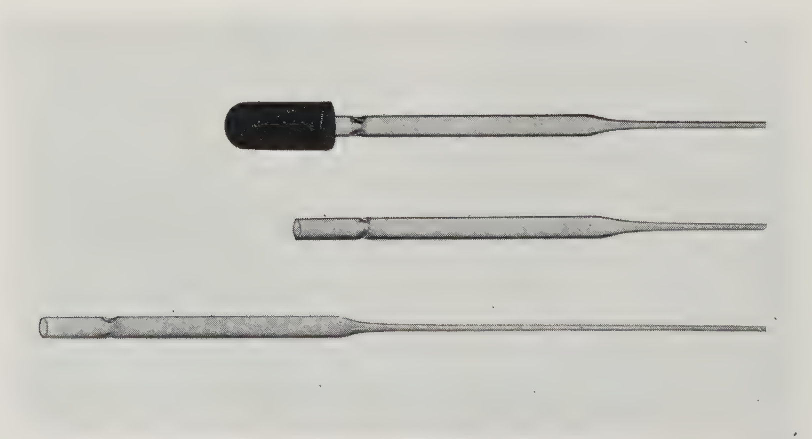

Fig. 468. diSPo-pipets. (Courtesy Scientific Products, Division of American Hospital Supply Corp., Evanston, Ill.)

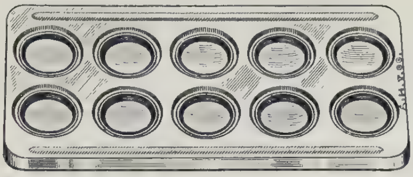

Fig. 469. Ten-welled slide for blood grouping. (Courtesy Arthur H. Thomas Co., Philadelphia, Pa.)

until ready for use. It warms to room temperature during the testing process.

Saline must be fresh, that is, preferably not more than 1 wk. old. Physiologic saline, 0.85% NaCl in distilled water, is used as the diluent in most of the tests. It should be filtered but need not necessarily be sterile. Chemical cleanliness is more important than sterility unless the diluted specimens are to be kept for some time in a refrigerator or unless the diluted blood is to be injected into animals or sent some distance from the laboratory.

Reagents: The following **testing sera** are needed for complete blood grouping tests for transfusions, including sensitivity testing for Rh-Hr antibodies, the A-B-O groups and M-N types, the antiglobulin (Coombs) test, and conglutination tests. All sera used for blood grouping work must meet the requirements for standards of potency (high titer) of the National Institutes of Health (NIH).*

1. Anti-**A** serum
2. Anti-**B** serum
3. Anti-**A**$_1$ serum (absorbed B or anti-**A**$_1$ Lectin)
4. Anti-**M** serum
5. Anti-**N** serum
6. Coombs serum (antihuman globulin)
7. 30% Bovine albumin
8. Anti-**Rh**$_0$ conglutinating (slide) serum
9. Anti-**Rh**$_0'$ conglutinating (slide) serum
10. Anti-**Rh**$_0''$ conglutinating (slide) serum
11. Anti-**hr**$'$ conglutinating (slide) serum
12. Anti-**hr**$''$ conglutinating (slide) serum
13. Anti-**Rh**$_0$ saline agglutinating (tube) serum
14. Anti-**rh**$'$ saline agglutinating (tube) serum†
15. Anti-**rh**$''$ saline agglutinating (tube) serum†
16. Anti-**hr**$'$ saline agglutinating (tube) serum†
17. Anti-**hr**$''$ saline agglutinating (tube) serum†
18. Witebsky group A and group B specific substances

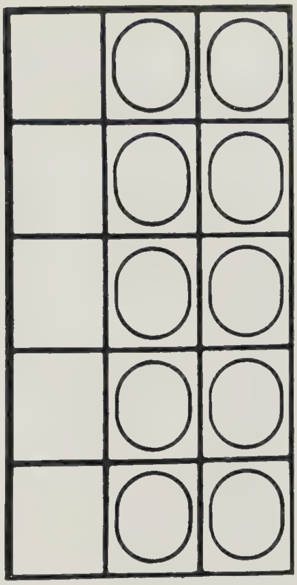

Fig. 470. Ceramic slide for blood grouping, with ovals and lines of red ceramics fused onto the glass. Sufficient space is allowed to number each specimen. (Courtesy Certified Blood Donor Service, Inc., Jamaica, L. I., N. Y.)

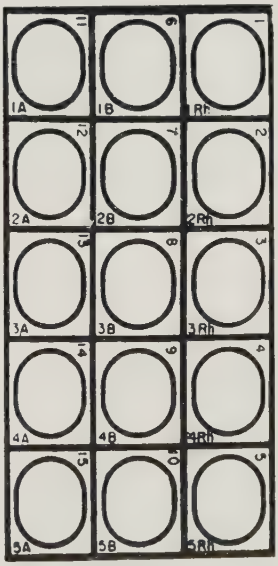

Fig. 471. Ceramic slide that permits large numbers of blood grouping tests at one time. This is the type of slide used in blood banks or in laboratories where a large number of such tests are performed at one time. (Courtesy Certified Blood Donors Service, Inc., Jamaica, L. I, N. Y.)

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*All blood testing sera, as well as whole blood and other fractions, are biologics under the Biologics Law. They must be licensed, prior to commercial distribution, by the National Institutes of Health, United States Public Health Service.

†If sera 14, 15, 16, and 17 are available, 9, 10, 11, and 12 may be omitted.

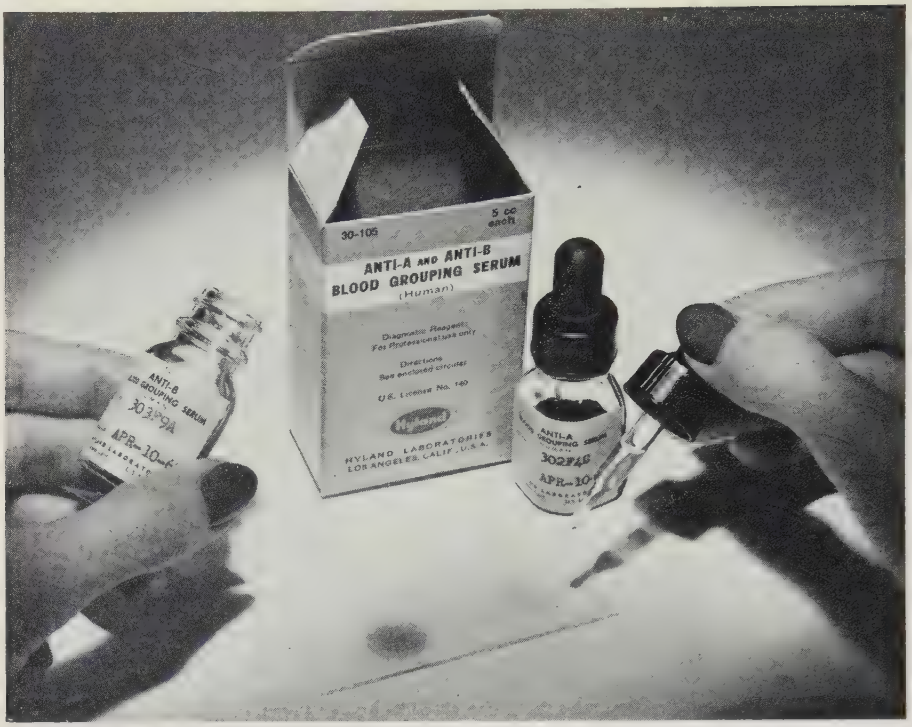
30-105
5 cc each
ANTI-A AND ANTI-B
BLOOD GROUPING SERUM
(Human)
Diagnostic Reagent
For Professional use only
Directions
See enclosed circular
U.S. License No. 140
Hyland
HYLAND LABORATORIES
LOS ANGELES, CALIF., U.S.A.
ANTI-B
GROUPING SERUM
303P9A
APR-10-6
ANTI-A
GROUPING SERUM
302F4S
APR-10

Fig. 472. Commercially available antisera for blood grouping tests. (Courtesy Hyland Laboratories, Los Angeles, Calif.)

The following **cell suspensions** must be prepared daily.* They are usable for 24 hr. Standard reference cell reagents, groups A_1 and B (human), are available from several sources.† These are specially prepared human blood cells containing a formula that eliminates hemolytic activity. They may be used as control cells or as the testing reagent when performing blood group tests on blood serum. In the latter case the serum need not be inactivated. If kept refrigerated, the cells maintain maximum reactivity for at least 3 wk. Control tests must be made using known anti-**A** and anti-**B** sera to make sure the cells have retained their activity. Most of the manufacturers add a dye as an additional precaution to prevent mix-ups. However, all laboratory workers should read labels carefully before use and should not depend on dyes in selecting their reagents. Before use, the cells should be thoroughly mixed in their respective containers.

Cell panels are also available for Rh-Hr testing. These are different red cell suspensions of group O blood tested for specific antigens. When the test cells are not purchased, the technician may prepare them in the laboratory. Prepare both saline-suspended untreated and trypsinated or ficinated cells of the various blood antigens for antibody tests, as follows:

Group O Rh_zRh_0
Group O Rh_1Rh_1
Group O Rh_2rh
Group O rh′rh
Group O rh″rh
Group O rh
Group A_1
Group B

The laboratory may prepare its own cells after testing individuals to determine the exact antigen on the cells; for example, group A_1 cells are obtained from an individual of group A_1, etc.

Additional reagents needed are as follows:

Trypsin, Difco 1:250, or ficin
Physiologic (0.85%) saline
Phosphate buffer, pH 7.41, M/15*

For the enzyme treatment of cells, some manufacturers now have available an acid solution of trypsin in Sørensen buffer.† This

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*In a small laboratory, if all the cells are not available, use group O Rh_z, O rh, A_1, and B cells.

†Certified Blood Donor Service, Inc., New York, N. Y.; Knickerbocker Biosales, Inc., New York, N. Y.; Hyland Laboratories, Los Angeles, Calif.

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*Eimer & Amend or other brand.

†Certified Blood Donor Service, Inc., Jamaica, L. I., N. Y.

may be used in place of the method outlined on p. 1463.

The use of ficinated cells has supplanted trypsinated cells in many laboratories.*

Equipment†:

1. Microscope containing a scanning lens, 32 mm. 0.10 strain-free. If this is not available, simply remove the front lens attachment from the low-power objective of a microscope and use the remaining lens system.
2. Electrically controlled water bath, which should be kept covered at all times.
3. An angle centrifuge (Figs. 473 and 474).‡
4. Racks for small tubes.§
5. Viewing box (Figs. 476 and 477).
6. A Brewer automatic pipetting machine is excellent but not absolutely essential.
7. Plain glass slides, 3 × 2 in.
8. 10-cell Boerner slides, 57 × 180 mm.‖ Fig. 469.
9. Ceramic 12-ring slides, containing baked-on red enamel rings (Figs. 470 and 471).
10. Wooden applicators.
11. Capillary or expendable droppers, and rubber bulbs.
12. Soda straws to use in place of droppers (or in addition to droppers).
13. Gauze pads, 3 × 3 in., 12 ply.
14. Red wax pencil.
15. Blue wax pencil.
16. Cover glasses, ⅞ in. square.
17. Tubes: 4 × ½ in.; 3 × ½ in.; 7.5 cm. × 8 mm.; B-D Vacutainer tubes for collecting specimens of blood.
18. Boerner electric rotating apparatus (Fig. 478).
19. Containers, about 250 ml. capacity, of different shapes, for holding saline and waste saline during the blood grouping tests.

Other reagents:

Preparation of saline: 0.85% NaCl. Strict chemical cleanliness is more important than absolute sterility in blood grouping serology.

Special anticoagulant for blood grouping:

Ammonium oxalate	3 gm.
Potassium oxalate	2 gm.
Distilled water	100 ml.

1. Put 0.2 ml. in the **bottom** of each tube. Dry in an oven.
2. For use, add 4.8 ml. venous blood and mix

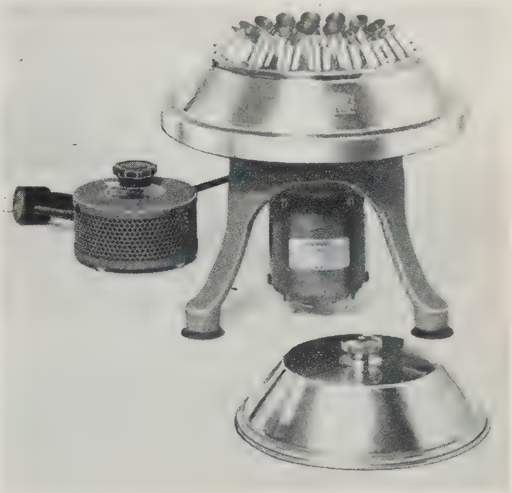

Fig. 473. Servall M, medium angle centrifuge. (Courtesy Ivan Sorvall, Inc., Norwalk, Conn.)

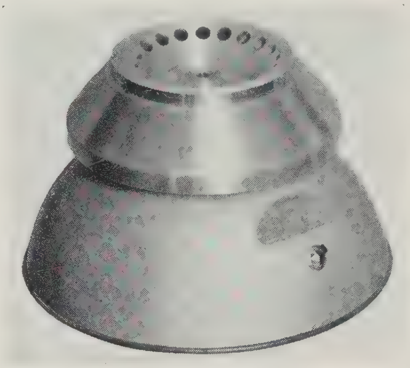

Fig. 474. Comet centrifuge. (Courtesy Phillips-Drucker, Astoria, Ore.)

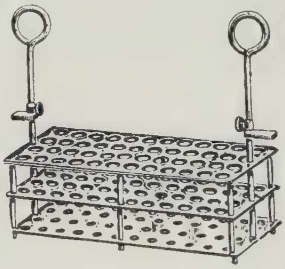

Fig. 475. Adjustable suspension test tube rack with handles, for small tubes used in blood grouping. (Courtesy Standard Scientific Supply Corp., New York, N. Y.)

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*Ficin is manufactured by Nutritional Biochemical, Cleveland, Ohio, and may be obtained from Fisher Scientific, Pittsburgh, Pa.

†The list of equipment used here was compiled from that used in the laboratory of Dr. Lester J. Unger in New York City (New York University Medical Center Blood Bank).

‡That made by Ivan Sorvall, New York, N. Y.; 27-tubed head, with 18 holders in the outer circle and 9 on the inner, is excellent. The Comet by Phillips-Drucker, Astoria, Ore., is also recommended.

§We recommend those made by Standard Scientific Corp., Catalog No. 95222, with 4 rows of 10 holes per row. The top and middle sections have ⅝ in. holes, whereas the bottom of the rack has small (5/16 in.) holes.

‖Arthur H. Thomas Co., Philadelphia, Pa.

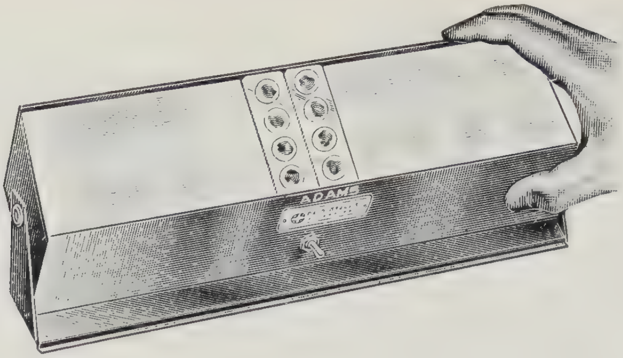

Fig. 476. Illuminated viewing box for blood grouping. (Courtesy Clay-Adams Co., New York, N. Y.)

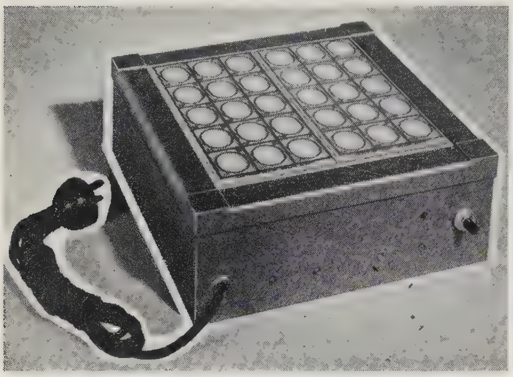

Fig. 477. Illuminated viewing box for blood grouping. (Courtesy Certified Blood Donor Service, Inc., Jamaica, L. I., N. Y.)

Thomas
ROTATING
APPARATUS
CONTINUOUS
SPEED
TIMER
A.H.T. CO.

Fig. 478. Boerner electric rotating apparatus. (Courtesy Arthur H. Thomas Co., Philadelphia, Pa.)

by inverting about 20 times to keep the blood from clotting. An excess of oxalate interferes with Rh testing, at times resulting in false negative results.

For smaller amounts of blood, use the following formula:

Ammonium oxalate . . .	1.2 gm.
Potassium oxalate	0.8 gm.
Distilled water	100 ml.

1. Place 0.1 ml. in the **bottom** of each tube and dry by gentle heat in a dry heat oven. Use 1 ml. of blood and proceed as above.

EDTA anticoagulant: The sodium and potassium salts of ethylenediamine tetraacetic acid, known as EDTA-S or EDTA-P, act as anticoagulants by forming nonionized calcium salts, thereby preventing the coagulation of blood. The dipotassium salt is recommended, although either may be used. EDTA is also known as Versene or Sequestrene.

1. Make a 0.5% aqueous solution by dissolving 0.5 gm. in 100 ml. distilled water. This solution contains 5 mg./ml.
2. For use place 0.2 ml. or 4 drops 0.5% solution in a small test tube and dry in an oven at 90°-100° C. In some tests the anticoagulant may be used in liquid form. Use the dry anticoagulant for blood grouping.
3. Add 1 ml. blood to the tube containing 1 mg. dry anticoagulant and mix by inverting 5 or 6 times. If larger quantities of blood are required, use a correspondingly larger quantity of the anticoagulant.

EDTA-P is available in 5 mg. tablets.* For use, place 1 tablet in a small tube, add 1 drop distilled water, then add 5 ml. venous blood, and mix by inverting 5 or 6 times.

Preparation of plasma or serum:

1. For plasma, take blood in the special anticoagulant or in EDTA.
2. Mix to prevent clotting.
3. Centrifuge or allow to stand until the cells settle.

Remove enough plasma for cross-matching tests, sensitivity testing, and any other work required. Serum is preferred to plasma for these tests.

Whenever possible, use serum, prepared in the usual manner from clotted blood. Leave enough serum on the clot to resuspend the cells (50% cells, 50% serum) for the protein-media (whole blood) tests. (See below and also Fig. 479.)

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*Cambridge Chemical Products, Inc., Dearborn, Mich.

Preparation of 2% or 3% cell suspension in physiologic saline (unknown cells):

1. Place about 1 ml. saline in a Kahn tube (3 × ½ in.).
2. Dip a straw into the cells left after removal of the plasma or serum, and add the cells to the saline, mixing in and out of the straw until a 2% or 3% suspension is obtained. This is judged by the color (tomato juice).
3. If clotted blood has been submitted, loosen the clot with a wooden applicator, centrifuge, remove the serum, use some of the cells shaken from the clot to make the saline suspension. Wash once in saline and resuspend to a 2% concentration with saline. Take care that no small clots remain in the cell suspension, since these might give a false appearance of clumping in the tests.
4. Another method of making a cell suspension from clotted blood is to dip a wooden applicator stick into the clot, mix around, and transfer the cells (which adhere to the stick) to saline in a small tube. Again, take care that no small clots remain in the saline suspension.

Preparation of control cell suspensions:

Untreated saline-suspended cells:

1. Prepare cell suspensions as outlined for the preparation of 2% or 3% cell suspension in physiologic saline.
2. Centrifuge to pack cells.
3. Remove supernatant saline.
4. Shake tubes to loosen cells.
5. Add saline to a 2% or 3% suspension (color of tomato juice) or 1 drop packed cells plus 30-49 drops saline. This is referred to as washing once with saline. Heavier suspensions are made for some tests.

Trypsinated saline-suspended cells: Trypsin must be prepared daily. Use Difco trypsin, 1:250. Use phosphate buffer solution, pH 7.41, M/15.*

1. Put a small amount of trypsin (about ¼ in. in a Wassermann tube) into a small tube, 5 × ½ in.
2. Add enough phosphate buffer to make a supersaturated solution.
3. Centrifuge. Remove the supernatant fluid and place it in another tube.
4. Wash a saline suspension of desired cells 4 times in saline.
5. Pack after the last washing by centrifuging; then remove the supernatant fluid.
6. To 9 drops packed washed cells add 1 drop freshly prepared trypsin.
7. Shake and place in a water bath at 37° C. for 1 hr., shaking every 15 min. while in the water bath.
8. Wash once with saline. (Add saline, centrifuge, and remove supernatant fluid.)
9. Resuspend to 5% concentration in saline, 1 drop concentrated cells plus 19 drops saline.

If kept refrigerated, trypsinated-saline suspended cells can be kept for 1 or 2 days. If some hemolysis occurs, wash once with saline and resuspend cells in fresh saline.

Ficinated-saline suspended cells:

1. Prepare a saline suspension of cells, wash twice with saline, and after the second washing centrifuge and remove the supernatant saline.
2. Prepare a 1% solution of ficin in phosphate buffer, pH 7.41, M/15: Place 0.1 gm. ficin in a Wassermann tube, and add 9.9 ml. phosphate buffer pH 7.41. Stopper and mix. This must **not** be a supersaturated solution. **Take care not to spill on the hands or splash into the eyes.** When the ficin has dissolved, centrifuge immediately to pack any undesirable particles or detritus and use the supernatant liquid.

If kept refrigerated, the ficin solution is useful for 5-6 days. If it is used often in the laboratory, it should be prepared at least twice a week.

3. Place 9 drops packed washed cells in a Wassermann tube.
4. Add 1 drop 1% ficin prepared as above.
5. Mix thoroughly and place in a water bath at 37° C. for 1 hr., shaking at intervals of 10-15 min. to make sure that all the cells are coated with the enzyme.
6. Fill the tube with saline and mix.
7. Centrifuge to pack the cells.
8. Remove the supernatant saline. This constitutes one washing.
9. Resuspend the cells to a 5% suspension.

These are enzyme-treated cells which are used in sensitivity testing and in any test for detection of univalent antibodies in the serum.

Enzyme-treated cells may be used for several days if kept refrigerated.

Rapid method of preparing ficinated cells—Wiener: The reagents, phosphate buffer, and 1% ficin are the same as those for ficinated-saline suspended cells.

1. Prepare a 3-5% suspension of the cells, wash twice with saline (centrifuge, remove supernatant saline, replace with a large volume of saline, and centrifuge; remove and discard the supernatant saline after the last washing).
2. Resuspend to a 3-5% suspension in saline.
3. Dilute the ficin solution 1:5 with saline, 1 part ficin solution to 4 parts saline.
4. Place 1 drop of the twice washed cells in a small Kahn tube and add 1 drop diluted ficin solution.
5. Mix by shaking and place in a water bath at 37° C. for 15 min.
6. Wash once with saline and remove and discard the supernatant fluid. This leaves 1 drop ficinated red blood cells in the tube.

Cells prepared in this manner are now ready to be tested with anti-**Rh**^{A}, anti-**hr′** serum, etc.

When the test calls for 2 drops of cells, use 2 drops of cells plus 1 drop diluted ficin solution and proceed as above.

When time is an important factor, it is best to use the rapid method of preparing ficinated cells for detecting unknown antibodies and for autoantibody tests on patients.

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*Eimer & Amend or other brand.

For routine work, group A_1, group B, group O (Rh_zRh_o*) cells are used; for titrations, group O Rh_1Rh_1, group O Rh_2rh, group O rh′rh, group O rh″rh, group O rh, or specific panel cells. See p. 1522.

Preparation of whole blood—protein media cells suspended in their own plasma (C_{50}), 50% suspension:

1. After centrifuging whole oxalated blood, remove part of the plasma until there are equal parts of plasma and cells.
2. Shake to resuspend the cells.

Whole blood is used for slide Rh testing, both with unknown and control blood, and for testing for A-B-O groups by the rapid slide method. Whole blood is not recommended for A-B-O tests.

Using droppers to maintain a standard drop: In order to maintain standard drops throughout a test and also to save time and equipment, the following method, as suggested by Unger, can be used:

1. Have 3 containers, approximately 250 ml. capacity, each of a different shape so as not to confuse them. One is for saline for use in the test, one is for rinsing saline, and one is for discarded saline, cells, and plasma.
2. Discharge material left in the dropper into the waste container, wiping the outside of the dropper with a gauze pad.
3. With the bulb still squeezed, pick up a dropperful of rinsing saline and discharge it into the waste container, and continue squeezing the bulb.
4. Wipe the outside of the dropper, and, still squeezing the dropper, pick up another dropperful of rinsing saline, discharge it into the waste container, and repeat 5 or 6 times. Be sure to wipe the outside of the dropper.
5. With the bulb still squeezed so as not to admit any material from the dropper into the clean saline, pick up a dropperful of saline from the saline for use, and use what is needed, discharging the excess into the waste container. Always hold the dropper at the same angle when measuring drops for a test. Do not admit bubbles from a dropper, for this would result in less than a full drop.
6. If handling serum or plasma by this method, discharge excess remaining serum or plasma from the dropper into the waste container and not back into the original specimen.

Preparation of slides for blood grouping, Rh testing, and cross matching:

Wax ring slides: Wax ring slides may be used. These are prepared as for the Kline test. Prepare the slides by cleaning thoroughly to render them fat-free and make wax rings with the usual contrivance furnished by the manufacturer, using hot melted paraffin to make the circles on the slide. The capacity of each ring is about 0.05 ml., so that small drops of serum and cell suspensions should be used. By using a tray that can hold 3 of the paraffin ring slides, as many as 36 tests can be performed at one time.

Boerner micro test slides: The Boerner micro test slides (Fig. 469) are an improvement over the wax ring slides. These test slides are 88 × 58 × 2 mm., with rounded corners, and have 10 mold-pressed, raised rings, 15 mm. top diameter, in 2 rows each, numbered consecutively from 1 to 10. The bottoms of the cells are flat and slightly rounded at the periphery, to prevent capillary flow around the outer edge and to ensure thorough shaking of the drop. They also permit rapid macroscopic and microscopic examination. Although designed for the Boerner-Jones-Lukens microflocculation test, the slides are satisfactory for blood grouping procedures.

Ceramic ring slides: These slides are also available from a number of manufacturers (Figs. 470 and 471). The ceramic rings are baked on the slides so that the slides may be used over and over.

When using whole blood (protein media), prepare slides by drawing a large oval with a red wax pencil, using a heavy line. Dimensions of the oval are 1½ × ¾ in. Ceramic ring slides are also available for these tests.

Electric rotating apparatus: Blood grouping tests are facilitated by using the Boerner electric rotating apparatus (Fig. 478) when using the open slide method. This apparatus provides perfect rotation of the slides. There is an automatic cut-off timing device with speed control rheostat and motor with worm reduction gear and driving mechanism that rotates the platform in a horizontal plane so that it circles an area ¾ in. in diameter to simulate hand rotation.

When testing for blood factors not usually searched for in routine procedures, other antisera and equipment are needed. These are listed with the individual tests.

Caution: When the antisera are not in use, they must be kept refrigerated.

Always protect the testing sera against bacterial contamination.

All work should be read by at least 2 technicians, without access to the records of each other. If there is any discrepancy in the results, the tests should be made again.

The cell suspensions used for the day's work should be prepared fresh daily at the same time, preferably by the same technician.

ROUTINE BLOOD GROUPING TESTS MADE ON PATIENTS AND DONORS (Figs. 479-482)

Tests other than those routinely used are described throughout the section.

1. Test for A-B-O group of both patient's and donor's cells (use donor's cells even though previously tested in the blood bank), using a 2% or 3% suspension of cells in saline against known anti-**A** and anti-**B** sera.

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*Agglutinated by all 5 standard antisera: anti-**rh′**, anti-**rh″**, anti-$\mathbf{Rh_o}$, anti-**hr′**, and anti-**hr″**.

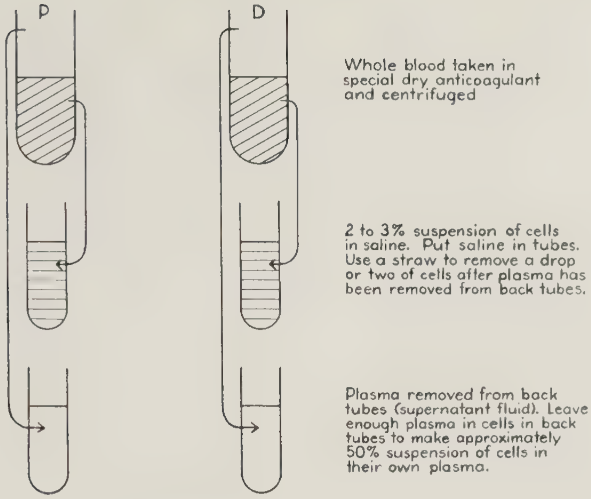
P
D
Whole blood taken in special dry anticoagulant and centrifuged
2 to 3% suspension of cells in saline. Put saline in tubes. Use a straw to remove a drop or two of cells after plasma has been removed from back tubes.
Plasma removed from back tubes (supernatant fluid). Leave enough plasma in cells in back tubes to make approximately 50% suspension of cells in their own plasma.

Fig. 479. Set-up for complete blood grouping of patient and donor. (After Unger.)

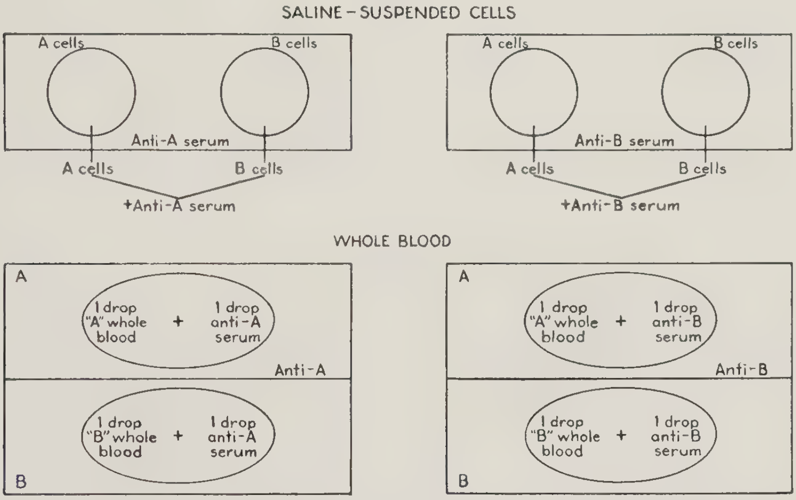
SALINE – SUSPENDED CELLS
A cells
B cells
Anti-A serum
A cells
B cells
+Anti-A serum
A cells
B cells
Anti-B serum
A cells
B cells
+Anti-B serum
WHOLE BLOOD
A
1 drop "A" whole blood + 1 drop anti-A serum
Anti-A
1 drop "B" whole blood + 1 drop anti-A serum
B
A
1 drop "A" whole blood + 1 drop anti-B serum
Anti-B
1 drop "B" whole blood + 1 drop anti-B serum
B

Fig. 480. Controls on anti-**A** and anti-**B** sera.

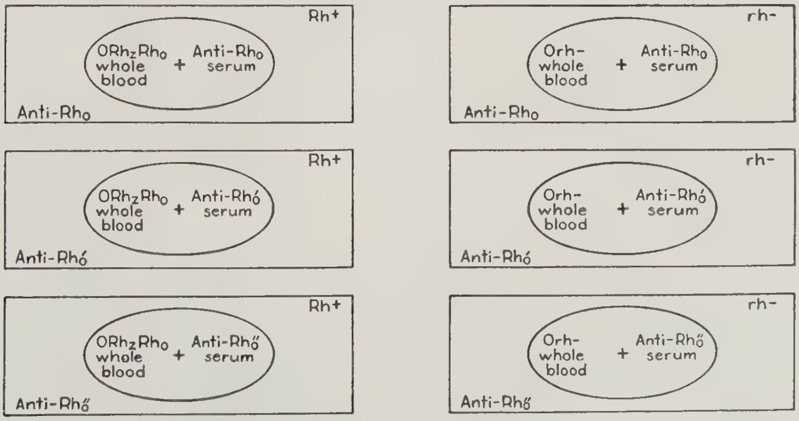
Rh+
ORh_zRh_0 whole blood + Anti-Rh_0 serum
Anti-Rh_0
rh-
Orh- whole blood + Anti-Rh_0 serum
Anti-Rh_0
Rh+
ORh_zRh_0 whole blood + Anti-Rh_0' serum
Anti-Rh_0'
rh-
Orh- whole blood + Anti-Rh_0' serum
Anti-Rh_0'
Rh+
ORh_zRh_0 whole blood + Anti-Rh_0'' serum
Anti-Rh_0''
rh-
Orh- whole blood + Anti-Rh_0'' serum
Anti-Rh_0''

Fig. 481. Controls on Rh antisera.

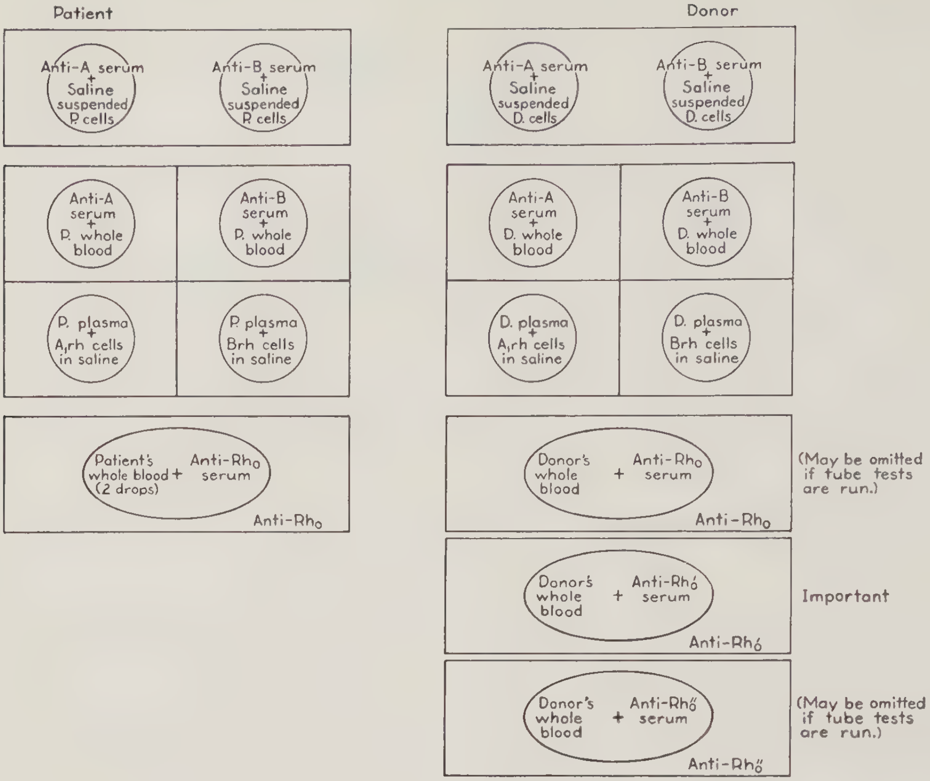
Patient
Anti-A serum + Saline suspended P. cells
Anti-B serum + Saline suspended P. cells
Anti-A serum + P. whole blood
Anti-B serum + P. whole blood
P. plasma + A$_1$rh cells in saline
P. plasma + Brh cells in saline
Patient's whole blood + (2 drops) Anti-Rh$_o$ serum
Anti-Rh$_o$
Donor
Anti-A serum + Saline suspended D. cells
Anti-B serum + Saline suspended D. cells
Anti-A serum + D. whole blood
Anti-B serum + D. whole blood
D. plasma + A$_1$rh cells in saline
D. plasma + Brh cells in saline
Donor's whole blood + Anti-Rh$_o$ serum
Anti-Rh$_o$
(May be omitted if tube tests are run.)
Donor's whole blood + Anti-Rh$_o'$ serum
Anti-Rh$_o'$
Important
Donor's whole blood + Anti-Rh$_o''$ serum
Anti-Rh$_o''$
(May be omitted if tube tests are run.)

Fig. 482. Tests on patient and donor.

2. At the same time, run controls on the antisera, using known group A$_1$ and group B cells (preferably but not necessarily type rh), 2% or 3% suspension in saline. The anti-**A** serum must cause agglutination of the A cells but not the B cells, and the anti-**B** serum must cause agglutination of the B cells but not the A cells.
3. Test the serum (or plasma) of both patient and donor against known group A$_1$ and group B cells suspended in saline. If isohemolysis occurs, inactivate the serum, cool to room temperature, and repeat the tests. If the results of this test differ from the results in step 1, repeat both tests, using a different set of known antisera to test the cell suspensions of patient and donor and different known A$_1$ and B cells to test their sera.
4. For further confirmation, test the whole blood of both patient and donor, using anti-**A** and anti-**B** sera, with the open slide method. This step is sometimes omitted from the routine procedure.
5. Subgroup all bloods belonging to groups A and AB by testing a saline suspension of cells against either absorbed B serum or anti-**A**$_1$ Lectin. Run suitable controls.
6. Test the **patient's** whole blood for the **Rh**$_o$ factor by the slide agglutination test, using anti-**Rh**$_o$ conglutinating (slide) serum. If the result is negative, consider the patient Rh-**negative** (even though there might be other Rh factors present), and use only **Rh-negative donor blood** for the transfusion. When possible proceed to step 8.
7. Test the **donor**'s whole blood against anti-**Rh**$_o$, anti-**Rh**$_o'$, and anti-**Rh**$_o''$ conglutinating (slide) sera.* If the blood gives a positive reaction with anti-**Rh**$_o$, all 3 tests will be positive. If the blood is negative with anti-**Rh**$_o$ serum but positive with either of the other 2 or with both, the donor must be considered as Rh-positive, and in such cases **do not give this blood to an Rh-negative patient.** If blood bank blood is used and tube tests have been made for the specific Rh factors, it is possible to find type rh (triple Rh-negative) donor blood. Any donor blood containing any of the Rh factors is unsafe for injection into an Rh-negative patient.
8. If the patient is Rh-negative by the slide test, and it is necessary to determine the exact Rh type, perform the Rh tube test, using 2% or 3% washed cell suspension in saline against known anti-Rh saline-agglutinating (tube) sera. Test for **Rh**$_o$, **rh'**, and **rh''**, as well as the Rh$_o$ variant ($\mathfrak{R}$**h**$_o$). If all these tests are negative, the patient is type rh (triple Rh-negative) and is assumed to be **hr'** and **hr''** positive. (For possible exceptions, refer to pp. 1501 and 1502.)
9. Cross match (a) the donor's cells against the patient's serum and (b) the patient's cells against the donor's serum, using saline-sus-

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*These antisera are discussed on p. 1514.

pended cells, incubating in a water bath, reading macroscopically and microscopically. If no agglutination is evident on microscopic examination, wash the cells and proceed with the antiglobulin test on the cell sediment. See step 11 below. If any agglutination appears, reject the donor.

10. Perform the open slide protein media (Unger) cross-matching test by using (a) donor's whole blood against patient's serum (fortified with albumin) and (b) patient's whole blood against donor's serum (fortified with albumin).
11. The conglutinin method of cross matching (Wiener) may be substituted for step 10 or used in conjunction with it.
12. At the same time that the cross-matching tests are made, make an Rh sensitivity test, using the patient's serum against group O Rh_zRh_o cells (cells that are agglutinated by anti-**Rh**$_o$, anti-**rh**′, anti-rh″, anti-**hr**′, anti-**hr**″ sera). Panel cells may be used. Perform these tests using (a) untreated, saline-suspended cells and (b) trypsinated or ficinated cells suspended in saline.
13. Similar sensitivity tests should be made on the donor's serum at the same time that tests are made on the patient's serum.
14. Test the patient's serum for evidence of autoantibodies. If desired, also test the donor's serum at the same time. The ficinated cell technic is used for this test. Use the patient's serum with the patient's cells and the donor's serum against the donor's cells.
15. If any of the cross-matching tests show positive results, reject the donor.
16. If the sensitivity tests or autoantibody tests on the donor's serum show positive results, reject the donor for all time.
17. If the patient's serum shows evidence of Rh-Hr antibodies or of autoantibodies, make suitable reports to the director of the laboratory or blood bank.
18. In all cases of pregnancy perform a sensitivity test on the mother's blood and, if positive, titrate for antibody concentration. Repeat the titration throughout the pregnancy as requested by the physician in charge.
19. In unexplained erythroblastosis fetalis, test the mother's serum for antibodies other than the Rh-Hr antibodies, as well as for specific Rh-Hr antibodies, and in the presence of an A-B-O incompatibility titrate for concentration of **A** and **B** antibodies, as well as for anti-C (of the A-B-O system). When the antibody cannot be identified, send the serum to a blood grouping serology specialist.

A-B-O SYSTEM

TESTING UNKNOWN CELLS TO DETERMINE BLOOD GROUPS

In testing for the A-B-O blood groups, at least 2 tests must be made on every specimen: (1) identification of the cells by use of anti-**A** and anti-**B** testing sera and (2) identification of the serum by use of known A_1 and B cells. In testing the cells, saline-suspended cells give clearest reactions. Whole blood is not recommended, although the method of testing whole blood is given below. Controls must be made whenever the unknown cells are to be tested.

Using saline-suspended cells

Controls

1. Have on hand glass slides containing 2 wax rings (Fig. 480).
2. Label the left side "A" cells and the right side "B" cells.
3. Label the first slide "anti-**A**" serum and the second slide "anti-**B**" serum.
4. Place 1 drop of A cell suspension (preferably A_2)* in the left-hand ring of each slide and 1 drop of group B cell suspension in the right-hand ring of each slide (see p. 1463 for preparation of cell suspensions).
5. Add 1 drop of anti-**A** serum to each ring of the first slide, and 1 drop of anti-**B** serum to each ring of the second slide. Take care that the tips of the droppers do not touch the cells.
6. Mix and rotate, examining macroscopically and microscopically under the low power, without the Abbé condenser, at the end of 5 min. If high-titer antisera are not available, wait a total of 8 min. before taking the initial reading. The use of any but high-titer antisera may lead to errors and therefore is not recommended.
7. Do not apply heat during performance of the tests.
8. Examine for agglutination or lack of agglutination. Agglutination is a positive reaction, and lack of agglutination is negative. Add a drop of saline to each ring, mix, and rotate thoroughly; then take a final reading under the microscope.

The results expected in the controls are given in Table 297.

Table 297. *Results expected in controls on anti-***A** *and anti-***B** *sera*

	"A" cell suspension	*"B" cell suspension*
Anti-**A** serum	+ (Agglutination within 45 sec.-4 min.)*	− (No agglutination)
Anti-**B** serum	− (No agglutination)	+ (Agglutination within 45 sec.-3 min.)*

*High-titer antisera usually cause clumping within 10 sec.

Agglutination must be +++S, and lack of agglutination must not be doubtful (see description below and on p. 1455). Anti-**A** serum must agglutinate the A cells but not the

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*More weakly reactive cells are used to show sera are potent.

B, whereas the anti-**B** serum must agglutinate the B cells but not the A.

If the proper results are obtained, grouping tests of the cells of the donor and the recipient may be undertaken, using these anti-**A** and anti-**B** sera. If agglutination does not take place properly or if the agglutinated masses are small, the antiserum is not usable. If there is no clear negative reaction, the antisera may not be used. In such cases discard the antisera and replace with new reagents, repeating the controls and the tests.

Actual blood grouping test using saline-suspended cells

1. Using a slide with 2 wax rings, label the left side "anti-A" and the right side "anti-B." Place the individual's name or laboratory number in the center of the slide for identification (Fig. 482).
2. Place a drop of the unknown cells in each ring.
3. Add 1 drop of anti-**A** serum to the left side and 1 drop of anti-**B** serum to the right side. Take care that the tips of the droppers do not come into contact with the cell suspensions. The antisera must not be contaminated in any manner. When the dropper is removed, hold the vial in a slanting position. When the antiserum is not in use, replace in a refrigerator.
4. Mix and rotate, examining macroscopically and microscopically (under low power without the condenser). Record a positive reaction whenever it occurs, but wait 5 min. before recording a negative. At the end of 5 min. add a drop of saline to each ring, rotate thoroughly to mix, and read again. Record agglutination as a positive reaction and lack of agglutination as a negative reaction. If there is any doubt about the reaction, repeat the test.

Some authors advocate mixing the contents of each ring with a separate toothpick or wooden applicator before rotating. If this technic is followed, take care to use a fresh applicator for each drop.

Do not apply heat. The action of the isoagglutinins is not enhanced by heat, and heat might actually interfere with the reaction.

Agglutination occurs as brick-red clumping of the cells, the clumps being completely separate from each other. Lack of agglutination is simply the homogeneous suspension of the cells as they appear when suspended in saline. Do not confuse settling of the cells and rouleaux formation of the red cells with true agglutination.

True agglutination is not affected by the addition of saline, whereas rouleaux are broken up with the addition of saline. See p. 1456 for a discussion of false reactions. Avoid working in drafts or where cold or warm air currents can blow over the open slides.

Determine the blood group by consulting Table 298.

Results of this test must be confirmed by testing the serum of the same individual against known A_1 and B cells as on p. 1469.

Table 298. *Identification of blood groups, using unknown cells*

*Unknown cells plus anti-**A** serum*	*Unknown cells plus anti-**B** serum*	*Group*
−	−	O
+	−	A
−	+	B
+	+	AB

− = no agglutination; + = agglutination.

Using cells suspended 50% in their own plasma (whole blood)

Controls

1. Prepare slides as described under the actual test below (Fig. 480).
2. Label the slides A cells + anti-**A**, B cells + anti-**A**, A cells + anti-**B**, and B cells + anti-**B**. Refer to Table 299.
3. Place about ½ drop of known A whole blood on the slides labeled "A" cells, and the same quantity of B whole blood on the slides labeled "B" cells.
4. Add 2 drops of anti-**A** serum to each slide labeled "anti-A" and 2 drops of anti-**B** serum to each slide labeled "anti-**B**."
5. Mix and spread each with a separate wooden applicator.
6. After sufficient time has elapsed for a reaction to take place, examine over a lighted viewing box (**not** preheated) for evidences of clumping. Add a drop of saline to each ring and permit it to run down through the drop of blood and serum mixture. Read after the addition of the saline. A final reading should not be taken in less than 5 min., and the blood must not be heated during that time.

Table 299. *Controls on anti-**A** and anti-**B** sera, using whole blood*

Slide	
1	Group A whole blood + anti-**A** serum
2	Group B whole blood + anti-**A** serum
3	Group A whole blood + anti-**B** serum
4	Group B whole blood + anti-**B** serum

Table 300. *Test of unknown whole blood for A-B-O group*

Slide	
1	Unknown whole blood + anti-**A** serum
2	Unknown whole blood + anti-**B** serum

These controls are used when whole blood is to be tested as in the method following.

The reactions should be the same as those in Table 297; a positive reaction should occur within 1 min. Do not read microscopically.

Actual test

1. Prepare slides, 3 × 2 in., by making red wax oval rings 1½ × ¾ in., 2 such rings on each slide. See Table 300.
2. Label one ring "anti-A" and the other, "anti-B."
3. Label with the name or laboratory number of the individual being tested.
4. Place the whole blood to be tested in each ring by just touching the slide with the wet straw so that only a fraction of a drop is placed on the slide.
5. Add 2 drops of anti-**A** serum (of very high titer) to its proper ring and 2 drops of high titer anti-**B** serum to its proper ring.
6. Mix each ring thoroughly, and spread each with a different wooden applicator.
7. Place on a viewing box and tip downward but do not heat the box. Allow to remain on the box for 5 min., rocking it back and forth occasionally. At the end of 5 min. turn on the light and read for clumping or lack of clumping.
8. With the box tipped downward add a drop of saline to the top of each ring and let it run down into the test. If rouleaux are present, the saline will break them up, but true agglutination will remain unaffected.
9. Determine the blood group by consulting Table 298.

If the results using the whole blood and the cell suspensions are the same, proceed with the test in which the plasma or serum is used as the unknown. If the results are different in the 2 tests, an error has been made, and the tests must be repeated.

The use of whole blood to determine the A-B-O groups is not recommended because the ratio of red cells to the antiserum is quite critical, and mistakes can be made easily when too much whole blood is used.

TESTING UNKNOWN SERUM OR PLASMA TO DETERMINE BLOOD GROUPS

This is a very important confirmatory test. It serves as a control over the results obtained when testing unknown cells. It is not recommended for use by itself to establish blood group of an unknown. When serum is being tested, it is not necessary to wash the A and B control cells, but these cell suspensions should be washed if plasma is used as the unknown.

In order to determine the blood group using serum or plasma as the unknown, it is necessary to have at hand cell suspensions in saline of known groups A_1 and B bloods, preferably but not necessarily type rh, suspended to 2% or 3% concentration.

If time permits, inactivate the serum at 56° C. for 30 min. to destroy complement and prevent subsequent hemolysis if the serum contains an isohemolysin. If an isohemolysin is present in a serum along with the corresponding isoagglutinin, the opposing cells begin to clump and then dissolve, and the reaction is difficult or at times impossible to read. Isohemolysins will not act except in the presence of complement, which is destroyed at 56° C. When the complement is destroyed, the isoagglutinin is unaffected, being thermostable, and the usual agglutination reaction takes place.

Actual test to determine blood group using unknown serum

1. Use a slide with 2 wax rings. Label the slide with the name or laboratory number of the unknown. Label "A cells" at the left side of the slide and "B cells" at the right side.
2. Place 1 drop of known A_1 cell suspension in the ring labeled "A cells" and 1 drop of the known B cell suspension in the ring labeled "B cells" (Fig. 482).
3. Add a large drop of the unknown serum (or plasma) to be tested to each ring.
4. Mix and rotate in the usual manner. Read a positive reaction as soon as it occurs, but do not take a final reading of a negative reaction until 8 min. have elapsed. Read all reactions both macroscopically and microscopically. Agglutination may be strong or weak, depending upon the concentration of antibodies in the unknown serum. Do not wait too long before reading, or surface drying takes place and pseudoagglutination may occur.
5. Add a drop of saline to each ring, mix again, and take a final reading under a microscope. Never permit the drops to dry.
6. Determine the blood group of the unknown by consulting Table 301.

Table 301. *Identification of blood groups using serum as the unknown*

A_1 cells plus unknown serum	*B cells plus unknown serum*	*Agglutinins in serum*	*Blood group*
+	+	Anti-**A** Anti-**B**	O
−	+	Anti-**B**	A
+	−	Anti-**A**	B
−	−	Neither	AB

Explanation: This is a test to determine anti-**A** and anti-**B** agglutinins in the unknown serum. Group O serum contains both anti-**A**, which agglutinates A cells, and anti-**B**, which agglutinates B cells. Group A serum contains anti-**B**, which agglutinates B cells but not A. Group B serum contains anti-**A**, which agglutinates A cells but not B. Group AB serum contains neither anti-**A** nor anti-**B** agglutinins and therefore cannot agglutinate either A or B cells.

If the results in this test differ from those obtained when testing the unknown cells against anti-**A** and anti-**B** sera, that is, if a different blood group is shown, repeat both tests but use different antisera and different control cell suspensions.

Caution: It has been noted by many workers that the anti-**A** and anti-**B** agglutinins in group O sera are not necessarily present in the same degree of potency in a single serum. Thus, when testing group O serum, the ring containing A_1 cells might show marked agglutination while the ring containing B cells might show but slight agglutination, or vice versa. In cases of subgroups, notably in subgroup A_2 or A_2B, the agglutinin content of the serum might be so exceptionally low that a false negative reaction could result. When, after repeated tests, using different cell suspensions as controls and different antisera for the tests against the unknown cells, the results of tests on the blood cells and blood serum of the same individual seem to give different blood grouping results, it is advisable to send the blood to a serology expert for further tests.

SUBGROUPS OF GROUPS A AND AB

Von Dungern and Hirszfeld[31] first called attention to the fact that when a group B serum was absorbed with certain group A bloods until it lost the power of agglutinating the absorbing blood, the serum still agglutinated most other bloods of group A and group AB. They suggested a possible subdivision of groups A and AB. In 1923 this was further emphasized by Coca and Klein[32] and resulted in the recognition of the fact that there are subgroups of A and AB, namely, A_1 and A_2 and A_1B and A_2B. Today other subgroups are recognized: A_3, A_4, A_5, and C_A (also called A_0 or A_x). These are divisions of agglutinogen A. In other words, in the sera of group B and group O individuals there are agglutinins that act on the agglutinogen A. According to the common concept this agglutinin is a single entity designated anti-**A**. Actually, the so-called alpha (α) or anti-**A** agglutinin is composed of a number of qualitatively different fractions, of which there are 2 main varieties: (1) anti-**A** agglutinin proper, reacting with both agglutinogens A_1 and A_2 with approximately equal intensity, and (2) agglutinin anti-A_1, which reacts with agglutinogen A_1 but practically not at all with agglutinogen A_2. Different group B and O sera contain these 2 agglutinin fractions in varying quantities and proportions.[33] Two different group B sera which react with A_1 cells at approximately the same titer may have different titers when tested against A_2 cells. If, for instance, such sera contain very much anti-$\mathbf{A}_1$ and very little of the common anti-**A** agglutinin, there might be a titer of 40 for A_1 cells and a titer of only 5 for A_2 cells. On the other hand, another serum which contains a large proportion of common anti-A agglutinin might have a titer of 20-40 for A_2 cells even though the titer for A_1 cells is no greater than that of the first serum. In some cases group B serum may react distinctly on A_1 and practically not at all on A_2 cells. In the case of AB blood, the danger of using such sera, just described, for testing purposes is great, since the sensitivity of A_2 agglutinogen in this group is greatly diminished and such blood is readily mistaken for group B. It is for this reason that every group B serum (anti-**A**) used for testing purposes should be titrated against A_2 as well as A_1 cells. Since A_2 makes up approximately ¼ to ⅕ of all group A individuals among Caucasoids, it is clear that with weak anti-**A** testing sera 20-25% of group A individuals may be erroneously diagnosed as group O.

Landsteiner and Levine showed that the subgroups A_1 and A_2 are determined by heredity.

While, as has been shown, bloods containing agglutinogen A differ in their sensitivity to clumping by anti-**A** sera, bloods containing B agglutinogen give uniformly strong reactions with anti-**B** sera. A knowledge of the subgroups is essential to explain puzzling phenomena which are occasionally observed in blood grouping and cross-matching tests.

In order to obtain the subgroups of A and AB, it is necessary to have at hand at all times blood cells belonging to known subgroups A_1 and A_2, which are used as controls. The antiserum used for determining the subgroups is an anti-**A** serum which has been absorbed with A_2 cells to remove the common anti-**A** agglutinin, leaving behind anti-$\mathbf{A}_1$. This antiserum is called "absorbed B" serum or anti-$\mathbf{A}_1$ serum. An anti-$\mathbf{A}_1$ reagent has also been prepared from the seeds of the *Dolichos biflorus*. This reagent is called "anti-$\mathbf{A}_1$ Lectin."* It is a vegetable hemagglutinin which contains a stable lectin with suitable anti-A_1 reactivity. In the commercial product 0.1% sodium azide is added as a preservative. We have used the anti-A_1 Lectin concurrently with absorbed anti-A serum on the same blood specimens over a period of many months and find it as suitable for use as the absorbed B serum. It has the property, as does the absorbed B or anti-A_1 serum, of causing agglutination of cells of groups A_1 or A_1B, but it does not clump cells of subgroups A_2 or A_2B.

False positive reactions occur when there is a residual trace of common anti-A agglutinin in the absorbed serum. On the other hand, if absorption of the common anti-A agglutinin is overzealous, the anti-A_1 agglutinin is also considerably weakened, and the serum is worthless.

If subgrouping tests are performed at too low a temperature, as in a cold room during winter, the residual nonspecific anti-A agglutinin will cause clumping of A_2 as well as of A_1 blood.

If the mixtures are allowed to dry because of delay in taking readings at the end of the prescribed time, false positive reactions may result.

Newborn infants' bloods may show false negative reactions. It is best not to report a final subgrouping reading until the infant is at least 3 mo. old.

False negative reactions occur when the room temperature is too high.

Old blood suspensions are less sensitive than freshly made suspensions, and, if used, false negative reactions might result.

In order to safeguard the subgrouping procedure against error, proper controls must be made using known A_1 and A_2 cell suspensions against the anti-A_1 serum, as explained below.

Technic of subgrouping A and AB blood cells

Controls

The reagent used for subgrouping groups A and AB cells, as stated above, may be either absorbed anti-A serum or anti-A_1 Lectin. When using an absorbed serum, it is necessary to limit the time between making the test and reading the reactions, since such sera are often slightly underabsorbed and may give false positive reactions after a prolonged time period. In order to determine the **time element** as well as to make sure the reagent reacts as it should, controls must be made just prior to using the reagent, testing it against known A_1 and A_2 cell suspensions.

1. Prepare 2-3% suspensions of known groups A_1 and A_2 cells, respectively, in saline. When using anti-A_1 Lectin, use a 10% cell suspension.
2. Place a drop of known A_1 cells in a wax ring on a slide properly labeled and a drop of known A_2 cells in another ring also properly labeled.
3. Add to each drop of cells 1 drop of absorbed anti-A serum or anti-A_1 Lectin.
4. Rotate and observe under the low power of a microscope until agglutination takes place, recording the time required to agglutinate A_1 cells. The A_2 cells must remain unagglutinated for at least 10 min. If the serum is not completely absorbed, they may eventually be agglutinated by the antiserum.
5. Record the time the first small clumps appear in the A_2 cell suspension ring if they do appear before the 10 min. time limit. The test on the unknown cells must be read within the time limit set by the controls.

For example, the A_1 cells were clumped in 1½ min., while the A_2 cells remained in even suspension with no clumps for 10 min. Allow a 2 min. time as a safety factor and read the results on the bloods being tested within 8 min. If the unknowns show agglutination in less than 8 min., the results may be recorded as positive. The time element is established primarily for reading negative results.

Actual subgrouping test

Using absorbed anti-A serum

1. Place 1 drop of the unknown cell suspension on the glass slide containing one wax ring, labeled with the name or number of the unknown and the group letter (A or AB).
2. Add 1 drop of absorbed anti-A serum and rotate after mixing.
3. Read microscopically under the low power without a condenser.
4. Take periodic readings, but record a negative reaction only after the end of the time limit

Table 302. *Determining subgroup of group A cells*

A cells plus absorbed anti-A serum	*Subgroup*
+	A_1
−	A_2

Table 303. *Determining subgroup of group AB cells*

AB cells plus absorbed anti-A serum	*Subgroup*
+	A_1B
−	A_2B

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*Hyland Laboratories, Los Angeles, Calif.

established by the controls. Record agglutination as positive as soon as it occurs.

If the cells have agglutinated, they are subgroup A_1 (or A_1B).

If they have not agglutinated, they are subgroup A_2 (or A_2B).

Tables 302 and 303 give these results in chart form.

Using anti-A_1 Lectin

Tube method:

1. Place 1 drop of anti-A_1 Lectin in a 10 × 75 mm. tube.
2. Add 1 drop of 2% cell suspension in saline.
3. Mix and centrifuge at 500-1000 r.p.m. for 1 min.
4. Read immediately after twirling the tube to dislodge the sedimented cells.

Subgroup A_1 or A_1B cells will remain in the original "button" as loosened by twirling. Subgroup A_2 or A_2B cells will form a smooth suspension.

Slide method:

1. Place 1 drop of anti-A_1 Lectin on a plain glass slide.
2. Add 1 drop of 10% suspension of cells in saline.
3. Mix and spread over an area of about 20 mm. diameter.
4. Tilt the slide from side to side and observe for macroscopic agglutination.

Clumping of A_1 or A_1B cells will be visible within 20 sec. and the reaction need not be observed for more than 1 min.

Cells of subgroup A_2 or A_2B will show no clumping during this period but may show a slight reaction if observed for a longer time.*

Agglutinogens A_3 and A_4

In 1935 Fischer and Hahn[34] reported the occurrence of rare individuals of group A whose blood gave only feeble reactions even with the most potent anti-**A** sera. Friedenreich, reporting on the same phenomenon, conceded that the weak reactivity of such bloods was due to a third sort of A agglutinogen, namely A_3. This agglutinogen A_3 gives rise to 2 additional subgroups in groups A and AB, subgroups A_3 and A_3B, respectively. These subgroups can be detected without any special method by their weak reactions with anti-**A** sera. A_3 blood, like A_2 blood, is agglutinated by sera containing anti-**A** isoagglutinin. Sera of group A_3 contain anti-**B** agglutinins just like ordinary group A sera, but, as in A_2 sera, anti-A_1 cold agglutinins may be present in these and in A_3B sera. The existence of A_3 receptor may account for certain errors in blood grouping.[35]

Wiener and Silverman[35] reported an interesting case belonging to subgroup A_3. Although this individual belonged to group A, the red cells showed weak and incomplete agglutination reactions with anti-**A** sera, both sera obtained from group B human beings and those prepared by immunizing rabbits with human A blood, in contrast to A_2 and A_1 cells which gave prompt and marked agglutination. Microscopic examination of the tests showed clumps on a background of many free cells. With weak anti-**A** sera the patient's blood might have failed to react. That she belonged to group A was further established by testing her serum against standard O, A_1, A_2, and B cells. With the B cells, prompt agglutination was obtained, demonstrating the presence of the anti-**B** agglutinin. No reaction occurred with O or A_2 blood cells, but a weak reaction occurred in the test with A_1 cells. This patient's serum contained, therefore, in addition to the anti-**B** agglutinin, an irregular agglutinin anti-$\mathbf{A}_1$. These observers therefore felt justified in placing this blood in the subgroup A_3.

An extremely rare A_4 blood group has been described by Gammelgaard and Marcussen.[36] There is a very weak reaction of the A_4 cells with anti-**A** sera. A further serologic characteristic is a reaction with certain group O sera and immune sera of rabbits, which is stronger than in group B sera. An irregular agglutinin for A blood cells was proved in the sera of 13 out of 15 cases. A_4 was found only once in routine examination of 60,000 blood samples. The presence of A_4 was proved in 24 persons of a family of 64 members. The importance of the A_4 group lies in a possible mistaken identification by reason of the very weak reaction, which is further lowered in combination with agglutinogen B as in group A_4B cells. The A_4 subgroup will rarely be revealed in routine serologic examinations of sera; therefore, in transfusions it may easily happen that blood of the A_4 group instead of O group is used. The A_4 group is comparable in its importance in legal medicine to that of the A_3 group, provided the hereditary process is clarified.

Factor C_A (A_o or A_x)—anti-C[37-41]*

In order to understand factor **C** of the A-B-O group, one must know the difference between an agglutinogen and a blood factor. An agglutinogen is a substance on the surface of the red cell. There are many agglutinogens on the human red cell shared by all members of the species and others that are type-specific, such as the A-B-O agglutinogens, the M-N-S-U agglutinogens, the Rh-Hr agglutinogens, etc. Agglutinogens are antigenic. When human cells are injected into a sensitive subject, antibodies are formed, which are capable of either clumping or lysing red cells of the same specificity as those injected to produce the antibodies. An agglutinogen can cause formation of and combine with more than one kind of antibody. The serologic specificities of agglutinogens defined by their corresponding antibodies are known as blood factors. Every agglutinogen has multiple blood factors that characterize it: e.g., agglutinogen M has the blood factors $\mathbf{M}_{i}$, $\mathbf{M}_{ii}$, $\mathbf{M}_{iii}$, etc.

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*Directions from pamphlet supplied by Hyland Laboratories, Los Angeles, Calif.

*Do not confuse factor **C** of the A-B-O system, mentioned by Moss in 1910, with Fisher and Race's "C" of the Rh-Hr system.

Agglutinogen A has 2 principal blood factors, **A** and **C**, whereas agglutinogen B also has 2 main factors, **B** and **C**. Corresponding to the 3 blood factors, **A**, **B**, and **C**, there are 3 naturally occurring agglutinins, anti-**A**, anti-**B**, and anti-**C**. Thus the composition of the 4 blood groups can be summarized as in Table 304.

Table 304. *Composition of the 4 blood groups*

Blood groups	*Red blood cells*		*Serum isoantibodies*
	Agglutinogens	*Blood factors*	
O	*	*	Anti-**A**, anti-**B**, and anti-**C**
A	A	**A** and **C**	Anti-**B**
B	B	**B** and **C**	Anti-**A**
AB	A and B	**A**, **B**, and **C**	None

*"O" is now considered a blood factor. Agglutinogen O and factor **O** are omitted here for the sake of simplicity.

If group O serum is titrated for anti-**A** and anti-**B** and then absorbed with cells of group A until it loses its ability to cause agglutination of group A cells, there is usually appreciable reduction in the titer for B cells. Conversely, if group O serum is absorbed by group B cells until it no longer agglutinates group B cells, the titer is also markedly reduced for group A cells. If, however, one pools anti-**A** and anti-**B** sera instead of using group O serum for the titration of antibodies and then absorbs such pooled sera with A cells, the anti-**B** titer is not reduced; nor is the anti-**A** titer reduced if such sera are absorbed with B cells. It thus seems reasonable that group O serum contains a third agglutinin, anti-**C**, specific for a blood factor **C** shared by agglutinogens A and B.

Moreover, injections of group A blood into group O individuals often cause a rise in titer for group B cells as well as for group A cells; similarly, injections of group B blood into group O subjects may stimulate a rise in the titer for group A as well as for group B cells.

It is relatively easy to prepare high-titered anti-**A** immune rabbit sera free of the **C** antibody, but anti-**B** immune sera are generally of considerably lower titer and contain a good deal of anti-**C** which is difficult to remove by absorption. Thus, if antisera from immunized rabbits are used for blood grouping, many group A individuals will be incorrectly classified as group AB because of traces of the anti-**C** agglutinin in the anti-**B** reagent, which will clump both A and B cells.

It is now apparent that a **blood group C,** with an incidence of 1 in 60,000 individuals, has been identified. This group is called C_A (formerly A_o or A_x); the cells fail to clump when mixed with anti-**B** or anti-**A** sera, but they are agglutinated by most group O sera. In a case cited by Wiener[33] the red cells reacted as group O in tests carried out in the usual manner with anti-**A** and anti-**B** grouping sera, whereas the serum agglutinated all cells of groups A and B but not of group O. In addition, the cells were agglutinated by all group O sera. Group O sera contain not only anti-**A** and anti-**B** but the agglutinin anti-**C**, not present in the sera of other blood groups, which seems to prove that this blood belonged to rare blood group C. The serum of this individual contained both anti-**A** and anti-**B** agglutinins.

It must be remembered that blood factor **C** is shared by both agglutinogens A and B and is therefore found in the cells of groups A, B, and AB but not in group O cells. In A-B-O hemolytic disease (p. 1539) the mothers are almost always of group O. If one postulates that most cases of A-B-O hemolytic disease are due to sensitization to the **C** factor, this would account for the excess of group O mothers in that disease.

The peculiar properties of group C_A appear to be due to a predominance of factor **C** on the red cells. The anti-**A**, **B** serum contains considerable anti-**C** and is therefore suitable for detecting bloods of group C_A. Refer to p. 1475 for use of this serum.

Subgroup A_m

According to Landsteiner's rule for the A-B-O groups, those agglutinins are regularly present in the plasma for which the corresponding agglutinogens are absent from the red cells, except during the neonatal period. In cases of agammaglobulinemia, the absence of agglutinins is merely one manifestation of a disorder which affects antibody production in general.

Wiener and Gordon[42] described a patient whose red cells reacted as group O and whose serum agglutinated group B cells only but failed to clump group A cells, even those of subgroup A_1. The gamma globulin concentration in the patient's serum was normal or slightly above normal. In tests with potent anti-**A** serum, not only from immunized human beings but also from immunized rabbits, the patient's red cells failed to agglutinate, which would indicate absence of blood factor **A**. Both groups A_4 and C_A (A_0) were excluded by further tests. Tests on the saliva showed that it inhibited anti-**A** strongly but did not inhibit anti-**B**, demonstrating that the saliva contained A substance.

The findings resembled the A-B-O reactions in lower monkeys and gorillas. Tests on monkey saliva disclosed the same reactions as this patient's saliva, and tests on the monkey cells were like those of the patient. Because the blood of this patient gave reactions resembling those of lower monkeys, the subgroup has been given the designation "A_m."

The suppressor *y* gene

The suppressor *y* gene, a very rare occurrence, inhibits the expression of the **A** antigen on the red cells but not in the saliva when it is present in its homozygous form *yy*. According to W. Weiner and others,[43] the **A** antigen could not be demonstrated on the red cells except by absorption. Less than normal amounts of A substance were present in the saliva in the case they described.

Factors of agglutinogen B

B_i, B_{ii}, B_{iii} – B_3: Although A and B agglutinogens are often treated as simple substances, it is well known that each is characterized by multiple factors. Agglutinogen B has a number of blood factors designated $\mathbf{B_i}$, $\mathbf{B_{ii}}$, $\mathbf{B_{iii}}$, all apparently found in most human groups B and AB blood. Factor $\mathbf{B_i}$ is not present in rabbit cells, the rabbit having only $\mathbf{B_{ii}}$ and $\mathbf{B_{iii}}$. Agglutinogen B also has factor **C**, as explained on pp. 1472 and 1473.

In 1955 Moullec et al.[44] described an unusual case in which the erythrocytes reacted very weakly with anti-**B** serum and absorbed the anti-**B** poorly. Eluates from these cells demonstrated more anti-**B** activity than did eluates prepared from normal B cells. The gene responsible for these reactions was traced through 3 generations. The saliva showed the presence of O (H) substance but the complete absence of B substance.

Sussman et al.[45] reported a second example of an unusual B_3 gene. During routine preoperative blood grouping and cross matching for transfusion they found that the serum gave different blood group results from those given by the cells. The patient's cells had been grouped as O, but the serum contained only anti-**A** and no anti-**B** agglutinin. This blood appeared to be compatible with group O blood donors not only in the tests using saline-suspended cells but also with the antiglobulin and high protein methods as well.

The patient's cells were agglutinated in potent anti-**B** sera and several group O sera after several hours at 25° C., but the agglutinates were barely visible clumps of 5-10 cells surrounded by many unagglutinated cells. When ficinated cells were used in cross matching, the results showed incompatibility with "immune" O sera and some random group O sera against the patient's cells, but all group B sera and some random group O sera were compatible.

Neutralization studies showed that the serum contained no demonstrable B substance. Although the saliva contained O (H) substance, no B substance was present. This discovery of another B_3 individual indicates the importance of testing the serum against known cells routinely in all blood grouping.

The "Bombay" blood type: The Bombay blood type was described in 1952 by Bhende et al.[46] and in 1955 by Levine et al.[47] The characteristics of this blood presumably result from the action of a pair of modifying genes *Xx*. In its rare homozygous form *xx*, the expression of the antigen **B** on the red cell and in the saliva is completely inhibited. The red cells of this blood type are not agglutinated by anti-**A**, anti-**B**, or several varieties of anti-**O** (H).[45]

The weak B of Mäkelä and Mäkelä[48]: These authors described a weak **B** antigen found on the red cells in the presence of an antibody of **B**-like activity in the serum. The patient's B cells were not agglutinated by the anti-**B** in the serum.

B_w of Levine, Celano, and Griset[49]: The weak B antigen $\mathbf{B_w}$ reacted with antiserum to produce the "extremely weak agglutination seen in artificial mixtures of 99 parts of group O blood and 1 part of normal group B blood." Both B and O (H) substances were present in the saliva of those carriers of $\mathbf{B_w}$ who were secretors. The $\mathbf{B_w}$ antigen is apparently weaker than $\mathbf{B_3}$.

$\mathbf{B_x}$ of Yokoyama, Stacey, and Dunsford[50, 51]: $\mathbf{B_x}$ was originally investigated because of the unexpected absence of anti-**B** from the serum of a presumably group O person. $\mathbf{B_x}$ is much weaker than $\mathbf{B_3}$. It reacts almost exclusively with immune O (anti-**A** and anti-**B**) serum. Small amounts of **B** antigen are demonstrable by absorption and elution studies on the red cells. B substance is clearly demonstrable in the serum and both B and O (H) substances are present in the saliva of all carriers of $\mathbf{B_x}$ gene. According to Sussman et al.,[45] the $\mathbf{B_x}$ of Yokoyama seems most nearly comparable to the $\mathbf{A_m}$ described by Wiener and Gordon.[42]

USE OF ANTI-A,B SERUM*

Anti-**A,B** serum is used (1) as a screening reagent to detect group O individuals who are to be used as "universal donors" and (2) as a confirmatory reagent in blood grouping tests.

Anti-**A,B** sera are obtained from group O individuals whose blood sera contain both anti-**A** and anti-**B** agglutinins in high titer and of high avidity. The anti-**A,B** serum will cause agglutination of cells of groups A, B, and AB but not of group O. Since, as previously stated, agglutinogen A contains a number of subgroups and bloods of these subgroups tend to react weakly with ordinary anti-**A** testing serum, the anti-**A,B** serum, being of high titer and avidity, reacts readily with blood containing the agglutinogen A_2 as well as with A_1. It also reacts with blood cells of the other subgroups of A.

Technic of using anti-A,B serum

Prepare a 2% suspension in saline of the blood to be tested.

Make the tests either in tubes with 7-8 mm. inside diameter or on the open slide.

Tube test:

1. Mix 1 drop of anti-**A,B** serum with 1 drop of 2% cell suspension in a small tube.
2. Permit to remain at room temperature for 1 hr. or centrifuge for 1 min. at 500 r.p.m.
3. Report clumping as positive and lack of clumping as negative.

Slide test:

1. Place 1 drop of 2% cell suspension in a well of a blood grouping slide.
2. Add 1 drop of anti-**A,B** serum.
3. Rotate back and forth for 10 min.
4. Read microscopically and macroscopically for agglutination or lack of agglutination.

If no agglutination occurs, the blood belongs to group O. If clumping occurs, the blood is A, B, or AB. Consult Table 305.

Table 305. *Reactions of unknown cells with anti-***A,B** *serum*

Cells of group	*Anti-***A** *serum*	*Anti-***B** *serum*	*Anti-***A,B** *serum*
O	–	–	–
A	+	–	+
B	–	+	+
AB	+	+	+

Suitable controls should be made of the anti-**A,B** serum; that is, the antiserum should be tested against groups A_1, A_2, B, and O cells. The controls are made in a manner similar to the tests.

PREPARATION OF ANTI-A AND ANTI-B TESTING SERA

Preparation of sera

The testing sera must be of a high degree of potency (titer). It is a mistake to use testing sera that do not meet the standards of the National Institutes of Health (N.I.H.). It is a mistake to pool different lots of anti-**A** or anti-**B** sera and use them for testing purposes unless each of the pooled sera is of high titer and avidity. No anti-**A** serum should be used for testing purposes unless it will agglutinate group A_1 cells at a 1:256 dilution, group A_2 cells at a 1:128 dilution, group A_1B cells at a 1:128 dilution, and group A_2B cells at a 1:64 dilution. No anti-**B** serum should be used to test unknown blood cells unless it will agglutinate group B cells at a 1:256 dilution. These are the requirements of the N.I.H. Unless one uses great quantities of the testing sera, it is much better to purchase them already prepared and standardized. Be sure to examine for the expiration date, and use only those manufactured under special license, in order to avoid grave errors in blood grouping tests.

Select an individual (group A for anti-**B** serum; group B for anti-**A** serum) with a high titer serum and without Rh antibodies. Use sterile precautions throughout the procedure of obtaining and preparing the serum. Remove the blood by venous puncture, either with a sterile 20 ml. syringe or, if blood is desired in larger quantities, use the commercially available vacuum bottles without anticoagulant. If using the smaller quantity, eject the contents of the syringe, under sterile precautions, into a clean, dry, sterile 50 ml. Rockefeller tube and immediately cork. Label with the name of the person from whom derived, the group letter, and the titer. Allow the blood to clot with the tube in a slanting position. Loosen the clot by striking the tube against the palm of the hand, and centrifuge at high speed for 30 min.

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*From a pamphlet by A. S. Wiener, New York, N. Y. By permission of Dr. Wiener.

Observing all sterile precautions, carefully remove the supernatant serum by means of a sterile dropper, placing it in a clean, dry, sterile tube labeled with the name of the person from whom derived, the group letter, and the titer.

Cork the tube immediately. Inactivate at 56° C. for 30 min. to destroy the natural complement to prevent subsequent hemolysis, and to kill any contaminating bacteria.

Transfer this serum with sterile precautions to sterile ampules and hermetically seal. Label with the name of the donor, the group letter, the titer, and the date. Store in a refrigerator.

Save enough of the serum to re-test before using. Test against groups A_1, A_2, A_1B, A_2B, and O cells, suspended in saline, and also use the whole blood. The anti-**A** serum must agglutinate the A_1, A_2, A_1B, and A_2B cells but not the B or O, whereas the anti-**B** serum must agglutinate the B, A_1B, and A_2B cells but not the A_1, A_2, or O cells. All positive reactions must be +++S and all negative reactions must be free of clumps. If any other results are obtained, the antiserum is not suitable for use as a testing reagent and must be discarded.

Titration of sera

It is necessary to titrate anti-**A** serum against A_1, A_2, A_1B, and A_2B cells, and anti-**B** serum against B cells, A_1B, and A_2B cells,* to avoid using antisera which are too weak for proper detection of the blood groups of unknown blood. Weakly reacting sera will give false negative results in the grouping tests, which may lead to serious transfusion reactions. Three examples of each group of cells should be used.

In all titrations, serial dilutions of each serum are made and each dilution is tested against the opposing cells. The reciprocal of the highest dilution of the serum that causes definite agglutination of its specific cells is called the titer. Thus, if the antiserum agglutinates the cells when it is diluted 1:256, the titer is 256. Many different methods of titrating sera have been proposed, but we prefer the method of Unger, which follows.

Making dilutions for titrations: The sera should be inactivated at 56° C. for 30 min. and allowed to cool to room temperature before dilution. Prepare serial dilutions of sera of 1:1 (undiluted) 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, and any other dilution desired or needed.

Use racks that hold 10 tubes per row. Rack up tubes 7.5 cm. × 8 mm., 10 per row, one row for each type of cells used. If titrating anti-**A** serum, 4 rows will be needed. If titrating anti-**B** serum, only 1 row will be needed, unless it is titrated against A_1B cells and A_2B cells, when 3 rows will be needed. These are the **titration tubes.** Refer to Fig. 496 for set-up for titrations.

At the same time, rack up 2 rows of Kahn tubes, 10 per row. These are the **dilution tubes.**

Use serum, not plasma, for titrations.

Place the serum in the first tube of the front row of Kahn tubes (dilution rack). Be sure that all tubes are properly labeled.

In all the back row tubes of the **dilution rack,** add saline to fill the tubes. This will be used to rinse the dropper. There are now 2 rows of dilution tubes, the first row empty except for the first tube, which contains serum, and a back row of 10 tubes filled with saline.

Put 1 drop of undiluted serum to be tested into the first tube of each row of the **titration tubes.**

Carry 10 drops of serum from the first tube to the second tube in the front row of **dilution tubes.**

Rinse the dropper with all the saline in tube 1 of the back row of dilution tubes and remove the discarded saline tube from the rack. Wipe off the excess saline with a gauze pad.

Put 10 drops of clean saline, using the same dropper at the same angle, in each tube of the front row of **dilution tubes,** beginning with tube 2.

Use the same dropper throughout the procedure and hold it at the same angle.

Mix the contents of tube 2 thoroughly in and out of the dropper, taking care to avoid bubbles.

Put a drop of this 1:2 dilution of serum into the second tube of the **titration racks** and carry 10 drops to tube 3 of the **dilution tubes.**

Rinse the dropper with all the saline behind tube 2 and wipe off the excess with a gauze pad.

Mix the contents of tube 3 (1:4 dilution) of the dilution tubes thoroughly and carry 1 drop to tube 3 of each **titration row** and 10 drops to tube 4 of the Kahn tubes for **dilutions.**

Again rinse the dropper, using the saline in the tube behind tube 3 and continue the dilutions and dispersal of drops as before until all the dilutions have been made and the titration racks contain 1 drop of the respective dilution per tube: 1:1 in the first tube, 1:2 in the second, 1:4 in the third, 1:8 in the fourth, 1:16 in the fifth, etc. See Fig. 496.

Add 1 drop of the correct cells* suspended in saline to each tube:

A_1 cells in one row containing serially diluted anti-**A** serum

A_2 cells in one row containing serially diluted anti-**A** serum

A_1B cells in one row containing serially diluted anti-**A** serum

A_2B cells in one row containing serially diluted anti-**A** serum

Use the same dropper after rinsing thoroughly with saline as directed. This ensures the same size drop for both serum and cells.

Shake thoroughly and leave for 2 hr. at room temperature. Tip the rack so that the bottoms of the tubes are visible and take readings from the bottoms of the tubes. Negative reactions run down

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*2% or 3% suspended in 0.85% saline (p. 1463).

*If anti-**B** (group A) serum is titrated against A_1B and A_2B, 3 rows of titration tubes will be needed.

Table 306. *Examples of titrations*

1:1	*1:2*	*1:4*	*1:8*	*1:16*	*1:32*	*1:64*	*1:128*	*1:256*	*1:512*	*Titer (units)**
++w	–	–	–	–	–	–	–	–	–	½
+++s	+++s	++w	+s	+w	–	–	–	–	–	16
+++s	+++s	+++s	+++s	+++s	+++w	+++w	++s	+w	±	256
+++s	+++w	+++w	++s	+	+w	–	–	–	–	32

*The titer (in units) is the reciprocal of the highest dilution of serum giving a 1 plus reaction.

in a pear-shaped formation. Agglutination reactions usually remain round.

Flick the tubes, gently spread the contents over the tube surface, and read under a scanning lens of a microscope. Or remove a small amount of sediment from each tube and examine under a microscope.

Reading results: It will be noted that the clumps of blood cells gradually become smaller as the serum becomes weaker. With some sera one dilution of serum may show strong agglutinating powers, whereas the next dilution shows a clear negative reaction. Report as 3 plus strong (+++S) any complete agglutination, that is, with no unagglutinated cells present. A 3 plus weak (+++W) reaction would be complete agglutination but with smaller clumps. A negative (−) reaction is absence of any signs of agglutination microscopically or macroscopically. Record reactions as +++S, +++W, ++S, ++W, +S, +W, ±, and −. The reciprocal of the last dilution in which there is definite agglutination (at least +W) is the titer.

Obviously, the first serum (Table 306) would be of no value for laboratory testing purposes. This blood would, however, yield ideal plasma because of the low isoagglutinin content. **Caution:** Even though a serum can be diluted and remain reactive, it is not useful for testing purposes unless it shows a +++S reaction when undiluted.

PREPARATION OF ABSORBED B (ANTI-A_1) SERUM

Observe all sterile precautions throughout the entire process. If the serum is contaminated, it will lose its agglutinating powers and will give false results.

Place a few ml. sterile 3.8% sodium citrate in a sterile 50 ml. Rockefeller tube and replace the cork immediately. Withdraw blood with a syringe from the vein of an individual of subgroup A_2 and add it with sterile precautions to the sodium citrate solution. It is best to pool the blood from a number of different group A_2 individuals.

Cork and mix well to prevent coagulation.

Centrifuge and wash 3 times with sterile 0.85% saline (p. 1463) and pack by centrifuging.

Discard the supernatant fluid after the last washing. These cells are the packed, washed, pooled group A_2 cells.

Place high titer group B (anti-A) serum, obtained under sterile precautions, in a 50 ml. sterile Rockefeller tube and inactivate at 56° C. for 30 min.

Add ⅓ to ¼ as much of the packed, washed, pooled group A_2 cells to the serum.

Cork and mix back and forth.

Allow the mixture to stand for 30 min. at room temperature, inverting occasionally; then centrifuge to separate the serum.

Remove and keep the supernatant fluid, which is absorbed B (anti-A_1) serum. It will usually be found that the recovered serum no longer reacts with group A_2 cells but still agglutinates group A_1 cells distinctly.

To test absorbed B (anti-A_1) serum

Have on hand a minimum of 3 known group A_1 and 3 known group A_2 cell suspensions derived from different individuals. These are prepared in the usual manner (p. 1463).

Place a drop of each cell suspension in a wax ring on a slide properly labeled and add a drop of absorbed B (anti-A_1) serum to each one.

Rotate and observe every 30 sec. under the low-power objective of a microscope until 5-10 min. have elapsed. A_1 cells should be agglutinated within 1 or 2 min., while the A_2 cells should not be agglutinated at the end of the 5-10 min. period.

If neither A_1 nor A_2 cells have been agglutinated, discard the serum. If the A_2 cells are agglutinated in less than 5 min., repeat the absorption, using fewer A_2 cells than during the preceding absorption period and reducing the time of absorption to guard against overabsorption. Test the serum against known A_1 and A_2 cells.

IRRADIATED BLOOD TYPING SERUM

With the advent of nuclear weapons and the common use of radioactive materials, a real possibility exists of exposure of medical biologicals to radiation. A study was made by Jamison to determine what change if any occurs when blood typing serum is exposed to ionizing radiation.[52]

Radiation causes coagulation and precipitation of protein in solution because of a denaturing process,[53] and this coagulation by radiation is irreversible. Changes have also been observed in surface tension and viscosity of protein solutions.[54]

Comparison of wet and dry typing sera irradiated at a dose level of 10,000 r with fresh nonirradiated typing serum revealed no change in the specificity of blood typing at the dose levels tested, and no delay was observed in the typing reaction. However, there did appear to be a slight acceleration in the rate of reaction of the Rh group with both wet and dry sera when the typing sera were exposed to 3000 r. Apparently blood-typing sera may be used after exposure to radiation in this dosage.

Rh TESTING

Before a cross-matching test is conducted, the blood of the patient must be tested to determine if it is Rh positive or Rh negative (usually for routine purposes determination of presence or absence of the **Rh**$_0$ factor is all that is required). Tests for the 3 principal Rh factors, **Rh**$_0$, **rh′**, and **rh″** are also made on the blood of the donor, and an Rh-negative donor is selected for the patient whose blood lacks the factor **Rh**$_0$. These tests are described on pp. 1514-1520.

TITRATION OF ANTI-C ("CROSS-REACTING ANTIBODY") IN GROUP O SERUM (USEFUL IN A-B-O ISOSENSITIZATION IN ERYTHROBLASTOSIS FETALIS)

Method of Jones et al.

Group O serum is characterized not only by the presence of anti-**A** and anti-**B** agglutinins but also by a third naturally occurring antibody, anti-**C** (p. 1473). As previously pointed out, absorption of group O serum by group A cells not only removes the anti-**A** agglutinins but also reduces the titer for group B cells; similarly, absorption with group B cells removes the anti-**B** agglutinins and reduces the titer for group A cells. Since both group A and B cells contain a mutual factor, "**C**," described by Moss in 1910, this reduction in titer of group O serum is due to removal of the naturally occurring anti-**C** of group O serum. In A-B-O hemolytic disease of the newborn it is the anti-**C** that coats the infant's cells.

Jones et al.[55] described a method of titration of group O serum for concentration of anti-**C** antibody, making use of the phenomenon of mixed agglutination of minor erythrocyte populations. If a suspension of group A red cells is diluted with a suspension of group O cells so that the concentration of group A cells is not greater than 1 part/500 parts of O cells, it is possible to sensitize the group A minor population cells with specific isoantibody, such as anti-**A**, without the occurrence of agglutination. If the suspension is washed to remove all free anti-**A**, addition of a further suspension of group A cells to the sensitized population mixture results in development of characteristic small agglutinates. Each of these agglutinates consists of an original sensitized A cell bound to the added or "detector" A cells by bivalent anti-**A** molecules. If one uses group O serum in place of anti-**A** serum to sensitize the group A minor population cells, specific mixed agglutinates will be formed not only on the addition of group A detector cells but also on the addition of group B detector cells. It thus appears that group O serum (but not group B serum) contains an antibody capable of uniting a group A cell to a group B cell.

The same type of experiment may be performed using group B cells instead of group A cells as the minor population and group A cells as the detector cells. Since group O serum contains both anti-**A** and anti-**B** in addition to anti-**C**, a group A minor population sensitized with group O serum will always be able to form mixed agglutinates with group A detector cells, and a group B minor population will also be able to form mixed agglutinates with a group B detector suspension.

The test consists of the attempted demonstration of the specific linkage of group A cells to group B cells through the medium of an antibody contained in group O serum. Those group O sera that have the power of linking A and B cells together are regarded as possessing anti-**C**, the cross-reacting antibody. The titration of this antibody is performed by determining the greatest dilution of serum at which this property can be demonstrated. As Jones et al. have pointed out, the titer is usually similar for both O (A) and O (B) population mixtures, although occasional examples of gross "asymmetry" are encountered.

It is possible that titrations for anti-**C** concentration in maternal serum may be useful in the prenatal diagnosis of erythroblastosis fetalis due to A-B-O incompatibility.

Cell suspensions: Prepare 5% suspensions of group A$_1$, group B, and group O cells by pipetting 2.5 ml. of twice-washed packed cells into separate 50 ml. volumetric flasks and diluting to 50 ml. with saline.

Population mixture O(A): Pipet 0.05 ml. of 5% group A cell suspension into a 25 ml. volumetric flask and dilute to 25 ml. with a 5% group O cell suspension.

Population mixture O(B): Pipet 0.05 ml. of 5% group B cell suspension into a 25 ml. volumetric flask and dilute to 25 ml. with 5% group O cell suspension. Invert both flasks repeatedly for at least 3 min.

Serum: Inactivate the serum to be tested at 56° C. for 20 min. Immediately before the test add the tetrasodium salt of ethylenediaminetetraacetic acid (EDTA) in a concentration of approximately 1 mg./ml. This suppresses anomalous mixed agglutinates, but the effect is reliable for only about 24 hr.

Isotonic EDTA-saline: Add EDTA to 0.9% sodium chloride solution in a concentration of 1 mg./ml.

Technic of the test:

1. Make 6 sets of serial 2-fold dilutions of the serum using EDTA-saline as the diluent, carrying out the dilutions to the tenth power (1:1024). See p. 1476 for correct method of preparing the diluted serum.
2. To each tube of the first 3 sets of dilutions add an equal volume of O(A) population mixture suspension.
3. To each tube of the second 3 sets of dilutions add an equal volume of the O(B) suspension. Refer to Table 307.

Table 307. *Cell population mixtures and added cells in Jones test*

Dilution set	*Population mixture*	*Group of added cells*
1	O(A)	A
2	O(A)	B
3	O(A)	O
4	O(B)	B
5	O(B)	A
6	O(B)	O

4. Mix by shaking and leave at room temperature for 15-20 min.
5. Wash the cell suspensions in all tubes 3 times in isotonic saline, taking care to avoid loss of cells. It is not necessary to use EDTA-saline for this step.
6. Restore to approximately the original volume.
7. Add equal volumes of group A, group B, and group O cell suspensions to each set of sensitized washed cells, respectively, A to the first series of dilutions, B to the second, and O to the third. Take care that the added cells are thoroughly interspersed in the population mixture by shaking and inversion. See Table 307.
8. Centrifuge the tubes at 250 g for 1 min.*
9. Gently but completely resuspend the cell deposit by inverting the tube.
10. Carry out the centrifugation and resuspension 3 times. Do not disturb the cell deposit after the final centrifugation until immediately prior to reading.

Reading results: Resuspend the cell deposit in sufficient saline to produce a convenient concentration of cells for observation in a hemacytometer chamber (about 0.2% concentration). Introduce the diluted suspension into the counting chamber and examine under a microscope for presence of characteristic agglutinates. The agglutinates are uniform spherical masses of cells about 20μ in diameter and occur with a frequency of about 2 per field.

The presence of typical mixed agglutinates in the suspension indicates a positive result. Absence of agglutinates is a negative result. There are generally fewer clumps in the last 2 tubes showing a positive result in the titration than in the suspensions exposed to more concentrated serum, but there is not the gradual diminution of clump frequency with progressive serum dilutions as seen in conventional agglutinin titrations. The end point of a mixed agglutination titration tends to be more sharply defined than the end point of an agglutinin titration.

The series of tubes to which group O detector cells were added constitute negative controls, and no agglutinates should be observed in those suspensions. If agglutinates are observed in the negative control tubes, this indicates lack of suppression of anomalous mixed agglutination or that the serum contains a specific or nonspecific agglutinin which is clumping the major population cells.

In this system, presence of anti-**C** in group O serum is revealed by formation of mixed agglutinates in those tubes in which a group B detector suspension has been added to an O(A) population mixture or in those tubes in which a group A detector suspension has been added to an O(B) population mixture. Addition of group A detector cells to an O (A) population mixture will always produce mixed agglutinates because of the almost invariable presence of anti-**A** in group O serum. Likewise those tubes in which group B detector cells are mixed with the O(B) population mixture should show mixed agglutinates.

The mixed agglutination titer of anti-**A** and anti-**B** in a group O serum will generally parallel the agglutinin titer. The titer of anti-**C** appears to bear only an approximate relationship to the agglutinin titers. Anti-**C** is more likely to be found in those group O sera having agglutinin titers for anti-**A** or anti-**B** over 256 and is less likely to be found when the agglutinin titer is below this level. According to Jones et al. tests of several hundred sera have not yet disclosed an instance of an anti-**C** titer in excess of those of anti-**A** or anti-**B**.

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*Consult the manufacturer's speed and force table for the centrifuge used or apply the equation: $RCF = 0.00001118 \times N^2 \times R$, where RCF = centrifugal force × g, N = revolutions/min., R = rotating radius in cm. at the outer tip of the tube.

TITRATION OF ANTI-A AND ANTI-B IN SERUM FOR DETECTION OF ISOSENSITIZATION IN ERYTHROBLASTOSIS FETALIS

Method of Unger modified after Wiener et al.

In cases of erythroblastosis fetalis or in sensitization unexplained by the Rh-Hr factors, it is necessary at times to titrate anti-**A** and anti-**B** in the patient's serum. With ordinary methods of titration it is not possible to differentiate between natural and artificially produced anti-**A** and anti-**B** antibodies. The following method attempts to make the differentiation.

Reagents:

Acacia:

1. Dissolve 20 gm. acacia (gum arabic) and 2 gm. Na_2HPO_4 (dibasic sodium phosphate) in 180 ml. distilled water.
2. Autoclave at 10 lb. pressure for 10 min.
3. Use the supernatant fluid.

Cells: Use A_1rh cells suspended in saline for titrating anti-**A** in group O or group B serum. Use Brh cells for titrating anti-**B** in group O or group A serum.

Prepare the cells in the usual manner, wash once with saline, and resuspend to a 2 or 3% concentration in saline. See p. 1463.

Procedure:

Titration for bivalent antibodies:

1. Make serial dilutions in saline of the serum to be tested: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512. See p. 1476 for exact technic.
2. Add 1 drop of the opposing cells to 1 drop of each dilution of serum. Carry out 2 titrations on group O serum, 1 using A_1rh and 1 using Brh cells.
3. Incubate in a water bath at 37° C. for 1 hr.
4. Examine the sediment macroscopically and under a scanning lens.
5. Report clumping as positive and lack of clumping as negative.
6. Record the result in each tube as the bivalent (agglutinating) antibody titer.

Acacia conglutination titer:

1. To each negative tube add 1 drop of the acacia solution.
2. Mix and incubate in a water bath at 37° C. for 1 hr.
3. Read as before. Report the results. This gives the acacia conglutination titer.

Titration for univalent antibodies:

1. At the same time run titrations on the same serum diluted 50% with Witebsky group specific substance (1:2).
2. Carry out the titrations exactly as directed above and report results in the same manner.

If there is a titer in this second set of tubes, the antibodies are due to isosensitization by A-B-O factors, since ordinarily Witebsky substance neutralizes bivalent anti-**A** and anti-**B** antibodies. Apparently it does not neutralize the univalent antibodies.

Examples: For examples, see Table 308.

Conclusions:

1. The titer for anti-**A** bivalent antibodies (Table 308) is 64. These are the naturally occurring antibodies.

2. The titer for anti-**A** univalent antibodies when acacia is used in the titration is 8, but there is no univalent antibody titer against saline-suspended untreated cells. These are the isoimmune antibodies.

3. The anti-**B** titer for bivalent antibodies is 128; when acacia (conglutinin) is used, it is 256.

4. The anti-**B** univalent antibody titer is 16 for untreated cells but 32 when acacia is added.

At birth, anti-**A** and anti-**B** are passively acquired by placental transfer from the mother. Anti-**A** and anti-**B** antibodies later

Table 308. *Titration of anti-***A** *and anti-***B** *bivalent and univalent antibodies*

	Dilutions										
	1:1	*1:2*	*1:4*	*1:8*	*1:16*	*1:32*	*1:64*	*1:128*	*1:256*	*1:512*	*Titer*
Group O serum, no Witebsky substance added, no acacia											*Bivalent*
1. A_1 cells	++s	++s	++	++w	+s	+s	+w	–	–	–	64
2. B cells	++s	++s	++s	++w	++w	+s	+s	+w	–	–	128
Same serum, no Witebsky substance, acacia-suspended cells											*Acacia (conglutinin)*
1. A_1 cells	++s	++	++	++w	++w	+s	+w	–	–	–	64
2. B cells	++s	++s	++s	++	++	++w	+s	+w	+w	–	256
Same serum, Witebsky substance added, no acacia											*Univalent*
1. A_1 cells	–	–	–	–	–	–	–	–	–	–	No titer
2. B cells	++s	++	++w	++w	+w	–	–	–	–	–	16
Same serum, Witebsky substance added, acacia-suspended cells											*Univalent*
1. A_1 cells	++	++w	+s	+	–	–	–	–	–	–	8
2. B cells	++s	++	++w	++w	+s	+w	–	–	–	–	32

developed by the individual are generally of heterospecific origin. The titration described here is used to differentiate between bivalent and univalent antibodies. This patient has been isosensitized against A and B agglutinogens and has univalent antibodies in the blood. If this occurs in unexplained erythroblastosis fetalis, the individual is sensitized to A-B-O and not the Rh-Hr factors.

CROSS MATCHING

After the blood group and Rh type of the patient have been established, and a donor of the same blood group and Rh type has been tentatively selected, cross-matching tests must be made. That is to say, one must match the donor's cells with the patient's serum and the patient's cells with the donor's serum, and there must be no signs of agglutination of the red cells. It is well to make sensitivity tests for Rh-Hr antibodies and tests for autoagglutinins at the same time as the cross-matching tests are run. See p. 1484 for the methods.

Caution must be exercised in accepting blood grouping designations by other laboratories. Remember that the person making the transfusion is responsible for any accident that may happen; therefore, all donors and recipients must be regrouped or retyped when they appear for examination or transfusion, and every patient or donor must be re-examined before every transfusion. This is in no way intended to criticize the work of other laboratories but is simply a measure of precaution which each one must follow.

One procedure, which is mentioned only to be condemned, is to perform cross-matching tests **alone,** omitting blood grouping. From time to time it has been claimed by some that cross-matching tests preliminary to transfusions are superfluous. It was erroneously believed that if the A-B-O blood grouping tests were properly carried out on the blood of the patient and donor, and if the results showed them to be of the same blood group, there would be no danger of a transfusion reaction. Unger found this to be incorrect.[56] He was the first to report that occasionally the serum of a patient would clump the cells of a donor even though they both belonged to the same A-B-O blood group and that transfusion of such blood was followed by chills and fever, which he attributed to the observed incompatibility (intragroup transfusion reactions). He called the antibodies responsible for the intragroup incompatibility "minor" agglutinins to distinguish them from the anti-**A** and anti-**B** agglutinins. He used a 2% suspension of the cells in the individuals own plasma to carry out these cross-matching tests, although at that time it was customary to use only saline-suspended cells. He had unwittingly carried out the "conglutination" test and detected the univalent antibodies (blocking, incomplete antibodies), although he did not identify them. Some of these most likely were Rh antibodies.

With the development of blood banks there has been a great increase in the number of patients transfused and the number of transfusions given to the same patient. These repeated administrations of blood expose patients to a great variety of antigens, and they may become sensitized, since it is practically impossible to transfuse a patient with blood that is identical to his own with respect to all blood factors. There are probably hundreds of thousands of possible combinations of blood factors known today. Perhaps some day a complete blood grouping may become as distinctive as a thumbprint, as predicted by Karl Landsteiner.

Correct cross-matching results can be obtained only on bloods of the same group or subgroup: while one may match the cells of the so-called "universal donor," group O, with the serum of almost every other individual, the reverse gives incompatible results; that is, the serum of group O will agglutinate the cells of all other groups—A, B, and AB, but not O.

Since cross matching is not a test for Rh types, it is necessary to make preliminary Rh tests on both patient and prospective donors.

Cross-matching tests must be capable of detecting not only the incompatibilities in the A-B-O system but must also detect univalent and bivalent antibodies in the other blood group systems. For this reason cross matching using saline-suspended cells only, without supplementary tests, is now considered definitely inadequate protection for the patient.

Technic of cross matching

Cells and serum are needed from the patient and from each prospective donor. The donor's serum may be obtained from the pilot tube if bank blood is used. The cell suspension from the donor may also be made from the pilot tube by dipping an applicator stick into the clot, breaking the clot, and transferring to saline the cells that adhere to the stick. If whole blood is to be used, it may also be obtained from the pilot tube, by preparing a suspension of cells in an equal volume of serum. Any serum remaining from the pilot tube may be replaced if the blood is not to be used at that time.

Cell suspensions are made in saline, using a 2% concentration (p. 1463). For the Unger protein media test use whole blood, that is, cells suspended to 50% concentration in their own serum or plasma. Oxalated whole blood, centrifuged and leaving enough plasma after decanting to make 50% plasma and 50% cells, is the best method of preparation.

A **routine cross-matching test** includes the following:

1. Donor's cells + patient's serum.
2. Patient's cells + donor's serum.
3. Patient's cells + patient's serum (the autoantibody test).

In addition, **sensitivity testing of the patient's and donors' sera** is carried out to detect the presence of antibodies:

1. Patient's serum + group O mixed antigen cells, cells untreated and saline-suspended.
2. Patient's serum + group O mixed antigen cells ficinated and saline-suspended.
3. Donor's serum + group O mixed antigen cells untreated and saline-suspended.
4. Donor's serum + group O mixed antigen cells ficinated and saline-suspended.

Tube method of cross matching (Fig. 483)

Saline agglutination test:

1. Use tubes 4 × ½ in. for cross matching.
2. Place in a rack in 2 rows. Two tubes are required for cross-matching one donor's blood with one recipient's blood. If more than one donor is available, run the 2 tubes for each donor.
3. Label the front tubes "PS" for patient's serum, and the back tubes "DS" for donor's

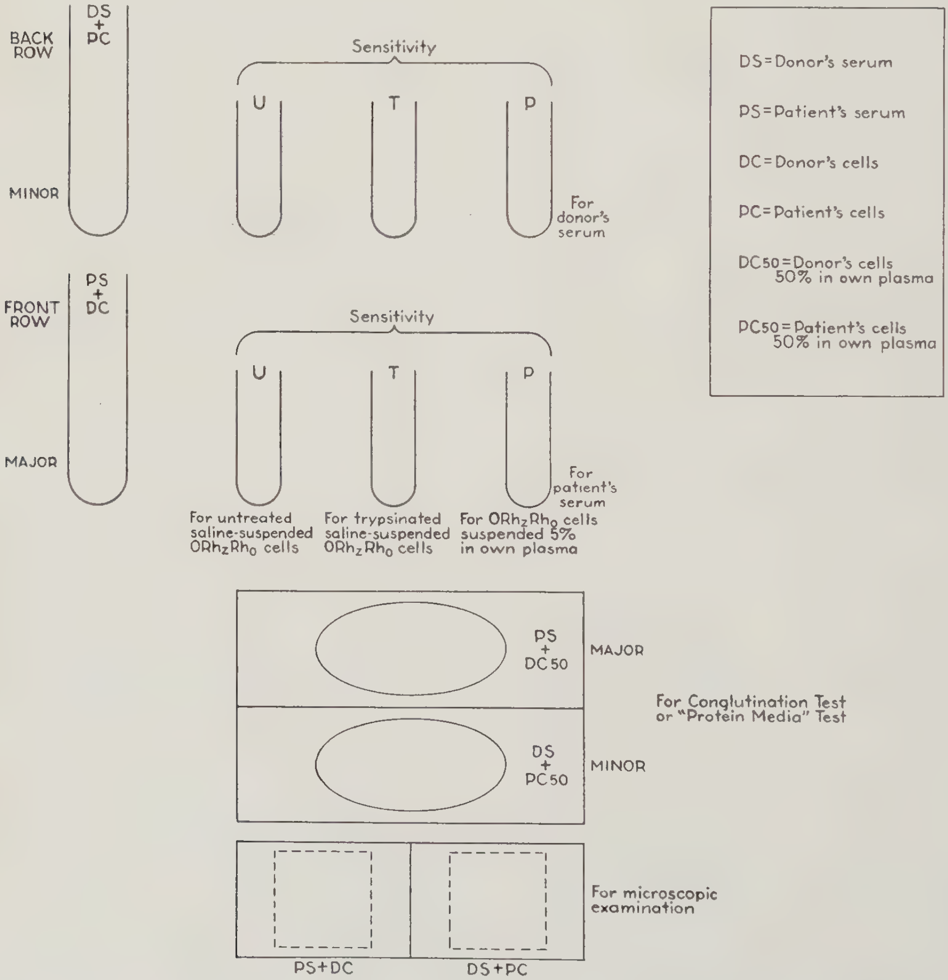
DS + PC
BACK ROW
MINOR
PS + DC
FRONT ROW
MAJOR
Sensitivity
U
T
P
For donor's serum
Sensitivity
U
T
P
For patient's serum
For untreated saline-suspended ORhzRho cells
For trypsinated saline-suspended ORhzRho cells
For ORhzRho cells suspended 5% in own plasma
DS=Donor's serum
PS=Patient's serum
DC=Donor's cells
PC=Patient's cells
DC50=Donor's cells 50% in own plasma
PC50=Patient's cells 50% in own plasma
PS + DC 50
MAJOR
DS + PC 50
MINOR
For Conglutination Test or "Protein Media" Test
For microscopic examination
PS+DC
DS+PC

Fig. 483. Cross matching and Rh-Hr sensitivity testing for antibodies in serum. (After Unger.)

serum. Identify each tube with the correct name or laboratory number.

4. Place 2 drops of patient's serum in the front tube (major).
5. Place 2 drops of donor's serum in the back tube (minor).
6. Add 2 drops of **donor's cells,** suspended in saline, to the front tube.
7. Add 2 drops of **patient's cells,** suspended in saline, to the back tube.
8. Mix each and incubate in a water bath at 37° C. for 30 min.
9. Centrifuge at 500 r.p.m. for 2 min. or 1000 r.p.m. for 1 min.
10. Pick up a small amount of the sediment from each tube with a straw or dropper and place on a properly labeled slide. Put a cover glass over each drop.
11. Examine under the low power of a microscope and search for clumping or lack of clumping.

If clumping of the cells has occurred, reject the donor. Examine the slides very carefully.

If the results are negative, examine the remaining contents of the tubes under a scanning lens of a microscope, by spreading the sediment the length of the tube with gentle rocking of the tube. If clumping appears, reject the donor.

This is a control over the slide reading.

If all the results are negative, proceed with the antiglobulin or Coombs test.

Coombs test (antiglobulin test): Coombs tests are made when the results in the cross-matching test (above) are negative.

1. Shake the tubes, add saline to the capacity of the tubes, centrifuge, and remove the supernatant saline. This is the first washing.
2. Shake the tubes, again add saline, and recentrifuge. Remove the supernatant saline and discard. This is the second washing.
3. Repeat for a third washing.
4. Remove the saline, turn the tubes upside down, and strike against a gauze pad to remove all traces of the saline from even the lip of the tube. Removal of all protein before the antiglobulin test (Coombs) is extremely important because if there is any protein left in the tube it interferes with and prevents an antiglobulin reaction.
5. Gently shake the tubes to loosen the cells.
6. Add 1 drop of antihuman globulin (Coombs serum) to each tube.
7. Mix.
8. Centrifuge at low speed, 500-1000 r.p.m., for 2 min.
9. Gently dislodge the sediment and read over a lighted viewing box and under a scanning lens of a microscope.

If any signs of clumping occur, reject the donor. See Table 309.

When the A-B-O compatibility has been proved, this test detects antibodies in the serum against one or more factors (other than A-B-O) of the blood it agglutinates.

Table 309. *Results possible in cross matching**

Donor's cells + *patient's serum*	*Patient's cells* + *donor's serum*	*Conclusions*
$\boxed{-}$ −	$\boxed{-}$ −	Compatible
$\boxed{-}$ −	$\boxed{+}$ −	Incompatible
$\boxed{+}$ −	$\boxed{-}$ −	Incompatible
$\boxed{+}$ −	$\boxed{+}$ −	Incompatible
$\boxed{-}$ −	+	Incompatible
+	$\boxed{-}$ −	Incompatible
+	+	Incompatible
+	$\boxed{+}$ −	Incompatible

*The box $\boxed{\ }$ was suggested by Unger to represent results of the antiglobulin (Coombs) test.

Slide method of cross matching, using whole blood—protein media method, Unger (Fig. 483)

The autoantibody test is included in this cross-maching method.

1. Prepare 3 glass slides, 3 × 1 in., by drawing on each a large red wax oval ring the length of the slide and half the width.
2. Label the first slide "PS," the second slide "DS," and the third slide "PS" + "PC."

   Slide 1:

   2 drops of whole blood of donor
   +
   1 drop of patient's serum
   +
   1 drop of 30% bovine albumin*

   Slide 2:

   2 drops of whole blood of patient
   +
   1 drop of donor's serum
   +
   1 drop of 30% bovine albumin

   Slide 3:

   2 drops of whole blood of patient
   +
   1 drop of patient's serum
   +
   1 drop of 30% bovine albumin
3. Mix the contents of each circle with a separate wooden applicator and spread each throughout the full surface of the ring. Place on a viewing box, turn on the light, and wait for 5 min. before taking a final reading. Do not preheat the box.
4. At the end of 5 min. tip the box and add a drop of saline at the top of each ring, allow-

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*Armour Laboratories, Chicago, Ill.; Tentex, Kankakee, Ill.; or Hyland Laboratories, Los Angeles, Calif.

ing it to run down through the specimen. Read for agglutination or lack of agglutination.

If either of the first 2 slides shows any evidence of agglutination, reject the donor. If there is no evidence of agglutination, the bloods are compatible and the donor's blood may be given to this patient.

If the autoantibody test is positive, that is, if the slide containing the patient's serum plus his own cells shows agglutination, the patient's blood contains autoantibodies. This must be reported immediately to the director of the laboratory. If this slide shows no agglutination, it may be considered a **negative control** over any positive reaction that may occur in the sensitivity test for antibodies in the patient's serum.

These slide tests are usually considered as screening tests; that is, if the results show incompatibility, there need be no further tests, but if the results are negative, then proceed with the tube test on pp. 1482 and 1484. These slide tests are never read microscopically.

Some laboratories prefer the conglutinin method of cross matching to the Coombs test. However, because the conglutination method cannot detect anti-**Fy**, the antiglobulin method must be used if an anti-**Fy** agglutinin is suspected.

Conglutinin method of cross matching

1. Carry out the test in 2 small Kahn tubes.
2. Place 1 drop of recipient's serum in a tube marked "RS."
3. Place 1 drop of donor's serum in a tube marked "DS."
4. Add 1 drop of 30% bovine albumin to each tube.
5. With an applicator stick, transfer enough cells from a clot of the donor's blood to make a 2% suspension of donor cells in the serum in the tube marked "RS."
6. Similarly, with an applicator stick transfer recipient cells to the serum in the tube marked "DS."
7. Incubate 3-5 min. at 37° C. in a water bath.
8. Centrifuge for 2 min. at 1000 r.p.m.
9. Dislodge the cell sediment very gently. Excessive shaking may break up agglutination. Observe for agglutination or lack of agglutination. If any agglutination is observed, reject the donor.

When A-B-O group compatibility is proved, agglutination in the tube marked "RS" indicates that the recipient is immunized against a blood factor of the donor cells. Further cross matching with other donor bloods must be carried out until blood is found that will not be agglutinated by the recipient's serum. Before testing with other donor bloods, the Rh type of the recipient must be carefully determined.

When Rh-positive recipients are immunized to Rh-positive blood belonging to a different Rh type and when Rh-negative recipients are erroneously designated Rh positive and isoimmunized, incompatibility with the donor blood may be detected by this method.

Routine test for cross matching, autoantibody testing, and detection of antibodies in serum (sensitivity testing), made simultaneously

When autoantibody and sensitivity tests are to be made simultaneously with cross-matching tests, 7 tubes are needed for each donor used with a specific patient.

In addition to 2% suspensions in saline of both the patient's and the donor's blood cells, saline suspensions of group O mixed antigen cells* and ficinated group O mixed antigen cells are needed.

Set up 7 tubes as follows for each donor used:

Tube 1: 2 drops of patient's serum + 2 drops of donor's cells (saline-suspended).

Tube 2: 2 drops of donor's serum + 2 drops of patient's cells (saline-suspended). Tubes 1 and 2 are for the cross-matching tests.

Tube 3: 2 drops of patient's serum + 2 drops of patient's cells (saline-suspended). Tube 3 is for the autoantibody test of the patient's serum.

Tube 4: 1 drop of patient's serum + 1 drop of group O mixed antigen cells, untreated and saline-suspended.

Tube 5: 1 drop of patient's serum + 1 drop of ficinated group O mixed antigen cells. If no mixed antigen cells are available, use group O type Rh_zRh_o cells, which have all the "standard" Rh-Hr blood factors.

Tube 6: 1 drop of donor's serum + 1 drop of group O mixed antigen cells, untreated and saline-suspended.

Tube 7: 1 drop of donor's serum + 1 drop of group O mixed antigen ficinated cells. Tubes 4 to 7 are for the sensitivity test (Rh-Hr and other antibodies in the serum).

Mix each tube separately, place in a rack, and put the rack in a water bath at 37° C. for 30 min.

Read macroscopically, observing the bottoms of the tubes. Positive reactions tend to remain in a "button," whereas in negative reactions the cells will run down when the tube is tipped and appear pear-shaped. Confirm all readings

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*Cells having **Rh₀**, **rh′**, **rh″**, **hr′**, **hr″**, and other blood factors. These may be prepared by the laboratory or purchased from Certified Blood Donor Service, Inc., Jamaica, L. I., N. Y., or Knickerbocker Laboratories, New York, N. Y.

by examining under a scanning lens of a microscope.

If clumping occurs, reject the donor.

If no clumping occurs, wash the contents of the tubes 3 times with saline in the usual manner, and after the last washing remove all traces of saline.

Add a drop of antiglobulin serum (Coombs) to each tube, mix thoroughly, centrifuge at 500-1000 r.p.m. for 2 min., and read. If clumping now appears in tube 1 or 2 or in both, the bloods of the patient and donor are incompatible and the donor must be rejected. If no clumping occurs, the bloods are compatible and this donor may be used for this transfusion.

If clumping occurs in tube 3, the patient's serum contains autoantibodies, and this must be recorded and reported to the director of the laboratory.

If any of the sensitivity tests, tube 4, 5, 6, or 7, show positive reactions, proceed in the usual fashion by reporting to the physician in charge and to the director of the laboratory, particularly if the donor's serum shows antibodies. Such donors must be rejected for all time.

Note: Unger[57] suggests that cross-matching tests must be made using patient's serum + donor's ficinated cells and donor's serum + patient's ficinated cells, using the tube method of cross matching when the sensitivity tests are positive by the ficinated cell technic and negative by the other methods used. When the reactions in such tests are positive, it is always necessary to rule out the possibility of the presence of autoantibodies in the patient's serum. Under such circumstances use the patient's serum + his own ficinated cells.

Sensitivity tests may be made either with group O mixed antigen cells or with group O cells containing only one particular antigen, but such cells are not always at hand. The commercial mixed antigen cells contain all the Rh-Hr factors as well as the rare factor **Fy^a**, along with **M, N, Kell,** etc. Both untreated saline-suspended and ficinated cells are used.

Anti-**Fy^a** will not react with enzyme-treated cells so that saline-suspended untreated cells must always be used when searching for the **Duffy** factor.

The Le^a, K, or P tests are carried out in a refrigerator at 4° C. and also at room temperature. If the cells are not enzyme treated, the refrigerator temperature must be used for the **Le^a** factor. It is customary to use untreated saline-suspended cells when testing for **Le^a** and **P** factors.

When the mixed antigen cells have been suspended in saline and kept refrigerated overnight, they should be washed twice with saline when collected and once again before use. Those that are to be used as untreated saline-suspended cells are retained in one tube, and those that are to be enzyme treated are removed and transferred to another tube after the last centrifugation, when the supernatant saline has been discarded.

When ficinating cells, it is best to use a 3-5% suspension, the 5% being preferred. The method of ficinating cells is given on p. 1463.

Rapid ficinated cell technic for autoantibody test:

1. Place 2 drops of patient's washed cells in a small Kahn tube.
2. Add 1 drop of 1:5 ficin (1% solution diluted 1:5 with saline), p. 1463.
3. Mix thoroughly and place in a water bath for 15 min.
4. Wash once and resuspend the sedimented cells to a 4% suspension (1 or 2 drops of saline).
5. Add 2 drops of patient's serum, mix, and place in a water bath at 37° C. for 30 min.

Read under a scanning lens. If clumping has occurred, the serum contains an autoantibody. If no clumping occurs, no autoantibody is present. Some authors suggest that an antiglobulin test be made at this point after washing 3 times with saline, but such a test might give false positive reactions, and it is not recommended by Wiener.

SIMPLE BLOOD MATCHING

Simple blood matching is not recommended since it assumes that the donor belongs to a blood group different from that of the recipient. According to older ideas, group O was called the "universal donor," since it was believed that it was safe to transfuse any individual with group O blood. Group AB was called the "universal recipient" because it was believed that it was safe to give blood of any individual to an AB recipient. The rule was expressed as follows: All individuals could receive blood from members of their own group and from group O, and all individuals could give their blood to members of their own group and to group AB patients. This is not true, and following such a rule can lead to dangerous reactions and even death.

Simple blood matching is still used, but it is not recommended. The only use for simple matching is if the patient is to receive blood from a group O donor, the patient belonging to some group other than O. The test is made by mixing the patient's blood serum with the donor's cells, following any of the cross-matching tests previously described (tube or slide) and observing for signs of agglutination. If any clumps are observed, the donor is rejected.

POSTTRANSFUSION REACTION "WORK-UP"

After a transfusion reaction it is necessary to perform certain tests to discover the cause. A complete A-B-O, Rh-Hr re-examination must be made, and in some cases tests should be made for antibodies against antigens other than those in the Rh-Hr system.

Test for all of the Rh factors and subgroups (refer to section on Rh types) and make sensitivity tests on the patient's serum against cells containing all the known antigens, but do not use pooled or mixed antigen cells. Also test the donor's serum against each of the antigens.

In making these tests use both the untreated saline-suspended cells and the enzyme-treated (ficinated) cells, except when testing for the $\mathbf{Fy^a}$ factor, because the **Duffy** factor is active only in untreated cells.

Test the sera of all donors used for this patient prior to the reaction, as well as the present donor. No matter how many transfusions have been given to the patient prior to the reaction, tests should be made of the bloods of all donors if they are still available.

Test to determine whether or not the patient's serum agglutinates the patient's own cells, particularly if this is an Rh_o-positive woman. This test should be made prior to the transfusion. If it has not been made, use a sample of the blood taken before the transfusion.

Perform a routine cross-matching test on both the pretransfusion and the posttransfusion specimens of the patient's and donor's bloods. To this routine add the ficinated cell technic. Ficinated cells are not usually used in the routine cross-matching tests unless so specified, but they are used when there has been a transfusion reaction. This cross-matching test after the transfusion reaction must be made on both pretransfusion and posttransfusion specimens.

If suspicions exist as to antibodies other than Rh-Hr, make studies on the pretransfusion blood of the patient for antibodies against all the antigens available, such as $\mathbf{Fy^a}$, $\mathbf{Le^a}$, etc.

Test the patient's cells to determine if the patient lacks any of the known antigens. In this case use antisera containing all the known antibodies and test against the patient's cells. If the patient lacks any of the known antigens as determined by this test, determine to which one, if any, of these factors the patient has been sensitized. See p. 1523.

Recapitulation

Test the patient's cells, using known antisera of various specificities.

Test the patient's serum against all the known antigen cells. For example, in a retransfusion, in which the patient is type Rh_1Rh_1, if he has been transfused with blood of type Rh_2, he could have developed antibodies of the specificities anti-$\mathbf{rh''}$ and anti-$\mathbf{hr'}$.

There are some cases on record of M-N sensitization. Tests must be made for such sensitization by testing the patient's serum against a series of both M and N cells in suspension in saline.

Additional tests

A **cell survival test** should be made. If the transfused cells survive and the patient has no demonstrable antibody, the reaction was due to some cause other than an incompatible transfusion.

In some cases in which the patient has had a transfusion reaction and tests indicate that the reaction was not caused by incompatibility, the survival tests are made. Use cells taken from the patient after the transfusion to determine whether or not he has any of the transfused blood. For example, if he is type M and has been transfused with type N blood, as long as the type N cells are still demonstrable in his blood, these cells have survived in the patient's circulation. In order to test one's own accuracy in determining the presence of small quantities of cells of type N, for example, in a patient's type M blood, mix various specimens, using 10 parts of type M and 1 part of type N bloods, or 5 parts of M and 5 of N, etc.; make tests in the usual manner, using anti-**M** and anti-**N** sera. Clumps of cells on a background of unclumped cells can be found in these experiments, which resemble those found in the survival test.

In the example just given, if an Rh_1 patient (blood cells have factors $\mathbf{Rh_o}$ and $\mathbf{rh'}$) has been transfused with $\mathbf{Rh_2}$ blood (with factors $\mathbf{Rh_o}$ and $\mathbf{rh''}$), and $\mathbf{rh''}$ positive cells are found in the patient's blood following the transfusion, then any reaction that took place is probably **not** due to anti-$\mathbf{rh''}$ agglutinins. If such agglutinins had been present, they would have destroyed the rh″ cells.

Chapter 74

The Rh-Hr system

ADDINE G. ERSKINE

Rh-Hr NOMENCLATURE[58]

Two principal methods of designation of the Rh-Hr types are currently in use, the original Rh-Hr nomenclature and the C-D-E symbols. Significantly, the nomenclatures interpret the same set of observations differently, and so both cannot be correct. The Committee on Medicolegal Problems* has analyzed the available information in order to resolve this problem. Their conclusion is that the C-D-E notations "make no allowance for the difference between a blood factor and an agglutinogen, and make the tacit incorrect assumption that every agglutinogen has but a single corresponding antibody. . . . The Committee therefore recommends that the C-D-E notations for the Rh-Hr types be discarded, and that the original Rh-Hr nomenclature be retained as the sole nomenclature for this blood group system."

Rh-Hr GLOSSARY[59-61]

antigen Any substance which, when introduced into the body, usually by a parenteral route, stimulates the formation of specific reacting substances.

antibodies Specifically reacting substances, believed to be globulins in chemical structure, produced by the body in response to the introduction of an antigen.

red cell antibodies Substances occurring in blood serum which react with their specific antigen or agglutinogen which are found on the surface of red cells. Some antibodies clump cells, some coat cells, some lyse cells, etc.

agglutinogen A substance on the surface of red blood cells which, in combination with its specific antibody (agglutinin), results in clumping of the cells. Agglutinogens are named for the most important blood factor or factors that characterize them. For example, agglutinogen A is named for blood factor **A**, although it shares factor **C** with agglutinogen B. (Agglutinogen A has a factor $\mathbf{F_A}$, which it shares with sheep cells.)

anti-rhesus serum In the narrow sense, antiserum prepared in rabbits, guinea pigs, goats, and other animals in response to the injection of blood of rhesus monkeys. Thus the term applies to sera like the original experimental serum of Landsteiner and Wiener, which clumps the bloods of 85% of all Caucasoids. In the broad sense, the term has been applied to include also all related Rh and Hr antisera of human origin.

isoimmunization; isosensitization Production of antibodies by an individual of a species in response to stimulation by an antigen derived from a different member of the same species.

rhesus factors; Rh factors; Rh agglutinogens; Rh antigens Attributes of the surface of red blood cells responsible for the reactions with Rh antisera. Study of anti-Rh sera of human origin shows that there are a variety of factors related to the original rhesus factor of Landsteiner and Wiener. The one corresponding to the original factor is designated $\mathbf{Rh_o}$. Reciprocally related factors are termed Hr factors. There are 4 standard Rh factors: $\mathbf{Rh_o}$, **rh′**, **rh″**, and $\mathbf{rh^w}$. Four Hr factors have been found: **hr′**, **hr″**, **hr**, and $\mathbf{hr^S}$. There are also minor variants of the Rh-Hr factors.

rhesus antibodies, Rh antibodies The specific substances responsible for the reactions produced by anti-rhesus sera. The anti-rhesus (anti-Rh) sera prepared by immunizing animals with rhesus blood give parallel reactions with human bloods. The anti-rhesus (anti-Rh) sera of human origin are of 8 principal varieties. (1) Rh antibodies: anti-$\mathbf{Rh_o}$, anti-**rh′**, anti-**rh″**, and anti-$\mathbf{rh^w}$. (2) Hr antibodies: anti-**hr′**, anti-**hr″**, anti-**hr**, and anti-$\mathbf{hr^S}$.

$\mathbf{Rh_o}$ factor; standard Rh factor This is the original Rh blood factor that corresponds to the original rhesus factor of Landsteiner and Wiener and is present in the blood of approximately 85% of Caucasoids, 95% of Negroids, and almost 100%

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*Medicolegal applications of blood grouping tests, supplementary report; A Report of the Committee on Medicolegal Problems, American Medical Association, prepared by A. S. Wiener, R. D. Owen, C. Stormont, and I. B. Wexler.[58]

of Mongoloids. It is the most antigenic of the Rh-Hr factors and therefore the most important clinically. More than 90% of cases of erythroblastosis fetalis and intragroup hemolytic transfusion reactions are due to this factor. When one speaks of the Rh factor, it usually is assumed that the **Rh**$_o$ factor is meant. The symbol for the factor **Rh**$_o$ is written with a large "R" to indicate that this is by far the most antigenic of the factors.

anti-Rh$_o$ serum; standard anti-Rh serum Serum specific for the **Rh**$_o$ factor. Only anti-**Rh**$_o$ serum of human origin is used for diagnostic purposes, since anti-rhesus immune animal serum clumps all blood specimens from newborn infants.

Rh testing Classifying human blood by tests with anti-**Rh**$_o$ serum alone. This divides all bloods into 2 types, Rh positive and Rh negative or, strictly speaking, **Rh**$_o$ positive and **Rh**$_o$ negative.

Hr factors Factors of Rh-Hr agglutinogens, which are responsible for reactions with Hr antisera. They are reciprocally related to the Rh factors, for which reason Levine first used the symbol "Hr" to indicate this relationship.

Hr sensitization The process of becoming sensitized to one or more of the Hr factors. When the Hr factor is not specified, it is usually the **hr**′ factor that is intended, since this is by far the most antigenic and the most important clinically.

Hr testing Examination of the blood with Hr antiserum. When the exact serum is not indicated, it is usually anti-**hr**′ serum that is intended.

Rh typing Classification of blood within 1 of the 8 Rh types with the aid of the 3 principal Rh antisera (see Table 310): anti-**Rh**$_o$, anti-**rh**′, and anti-**rh**″. There are 4 **Rh**$_o$-positive types and 4 **Rh**$_o$-negative types. For blood transfusions to all Rh-negative recipients, only type rh (Rh negative by the use of all 3 Rh antisera) should be given, to avoid isoimmunization due to injection of blood containing **rh**′ or **rh**″ factors. **All recipients who belong to types rh′ or rh″ must be given Rh-negative blood** (type rh*); since they lack the most antigenic of the Rh factors, **Rh**$_o$, they must not receive **Rh**$_o$-positive blood in a transfusion. **All donors who are Rh$_o$-negative but rh′- or rh″ positive must give their blood only to Rh-positive individuals.**

univalent antibodies One form in which antibodies of any given specificity may occur. Such antibodies tend to appear usually late in the course of immunization, are relatively thermostable, and readily pass through the intact placenta. In saline media univalent **Rh**$_o$ antibodies combine with and coat Rh-positive cells without clumping them. In protein media or in the presence of high-molecular colloids, univalent Rh antibodies clump cells containing the corresponding specific blood factor.

bivalent antibodies Another form in which antibodies of a given specificity may occur. Such antibodies usually are produced early in the course of immunization, are relatively thermolabile, and are effectively held back by the intact placental barrier. In saline as well as colloidal media they are capable of directly clumping cells possessing the corresponding specific antigen.

The differences between the bivalent and the univalent antibodies are summarized in Table 311.

agglutination Specific clumping of cells produced by bivalent antibodies.

conglutination Specific clumping of cells produced by the combined action of univalent antibodies and conglutinin.

conglutinin A colloidal aggregate of serum proteins which is adsorbed by cells that have been sensitized by their specific univalent antibody but not by cells that have not been sensitized and, after adsorption, causes the cells to stick together. Conglutinin is relatively thermostable but is sensitive to dilution with crystalline solutions, which causes it to dissociate into its constituent molecules. It is related to complement.

conglutinin substitutes Surface colloids, such as acacia, gelatin, dextran, and PVP, of complex molecular structure which can take the place of plasma in the conglutination test. Their ability to produce conglutination is due to their cohesive and adhesive properties, but they have the disadvantage of causing cells not coated by univalent antibodies to clump weakly, an undesirable quality not exhibited by natural or fortified conglutinin, and therefore it is not advisable to use these substitutes in routine practice.

fortified conglutinin Conglutinin that has been improved by the addition of the optimal amounts of albumin. Appropriate amounts (¼-⅓ its volume) of 30% albumin mixed with plasma contribute to the colloidal aggregates of protein in the plasma and thus enhance the conglutinin effect.

Rh antisera, anti-Rh sera, anti-rhesus sera Sera containing Rh or Hr antibodies, or both. These antibodies may occur singly, such as anti-**Rh**$_o$, anti-**rh**′, anti-**rh**″, anti-**rh**^{w}, anti-**hr**, anti-**hr**′, anti-**hr**″, and anti-**hr**^{S}, or in combinations, such as anti-**Rh**$_o$′, anti-**Rh**$_o$″, anti-**Rh**$_o$′″, anti-**rh**″,**hr**′, anti-**rh**′,**hr**″, and anti-**hr**″,**hr**^{S}.

Rh agglutinating serum Serum containing Rh antibodies predominately of the bivalent variety. Such antisera usually give best results by the so-called tube agglutination method in which dilute saline suspensions of red cells are mixed with the testing serum in small tubes and the reactions read after incubation at body temperature for 60-120 min.

Rh conglutinating serum Serum containing Rh antibodies predominately of the univalent variety. Such sera give best results by a variety of technics utilizing the principles of conglutination. For example, in the slide method a drop of serum is mixed with a drop of the whole, undiluted, oxalated blood being tested and the reaction read with the naked eye after the slide has been tilted back and forth for 1-2 min. over a warmed viewing box. Tube technics may also be used, for example, by using diluted suspensions of cells in plasma or albumin instead of saline or the indirect antiglobulin (Coombs) test. It is possible that all Rh antisera contain a mixture of both univalent and bivalent antibodies. If the univalent antibodies predominate, the antiserum must be

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*If blood of the same rare type as that of the patient (rh′ for an rh′ patient, etc.) is available, it may be used.

Table 310. *Scheme of the 8 principal Rh blood types*

Blood containing factor **Rh$_o$** (**Rh$_o$** *positive*)				*Blood not containing factor* **Rh$_o$** (**Rh$_o$** *negative*)			
*Designation of types**	*Reactions with sera*			*Designation of types**	*Reactions with sera*		
	Anti-**rh′**	*Anti*-**rh″**	*Anti*-**Rh$_o$**		*Anti*-**rh′**	*Anti*-**rh″**	*Anti*-**Rh$_o$**
Rh$_o$	–	–	+	rh	–	–	–
Rh$_1$	+	–	+	rh′	+	–	–
Rh$_2$	–	+	+	rh″	–	+	–
Rh$_z$	+	+	+	rh$_y$	+	+	–

*Type Rh$_1$ contains the 2 principal factors, **Rh$_o$** and **rh′**, the name Rh$_1$ being short for Rh$_o'$; similarly Rh$_2$ is short for Rh$_o''$ and Rh$_z$ is short Rh$_o'$Rh$_o''$.

Table 311. *Properties of univalent and bivalent antibodies**

Characteristic	*Bivalent antibodies*	*Univalent antibodies*
Common name	Agglutinin, precipitin, agglutinating antibody, complete antibody	Glutinin, blocking antibody, conglutinating antibody, incomplete antibody
Usual time of appearance in course of immunization	Early	Late
Resistance to heating	Relatively thermolabile	Relatively thermostable
Reactions of **Rh$_o$** antibodies† in saline medium	Clumps cells by agglutination	Coats cells without clumping them—blocking reaction
Reactions of **Rh$_o$** antibodies in colloid medium	Clumps cells by agglutination	Clumps cells by conglutination
Reaction in presence of complement	Does not fix complement or lyse cells	Fixes complement and lyses cells if number of antigenic loci on cells surface is adequate; viz., lysis occurs in A-B-O tests but not in Rh-Hr, Kell, or Duffy tests
Behavior in mixed agglutination tests	Specific clumps are formed	Clumps contain more than one kind of cell
Opsonic effect	None	Positive in presence of complement
Chemical nature	Euglobulin; precipitated by sodium sulfate solutions of concentrations 13.5-17.4%	Pseudoglobulin; precipitated by sodium sulfate solutions of concentrations 17.4-21.5%
Electrophoretic behavior	Alpha and beta globulins	Gamma globulins
Sedimentation constant	17	7
Probable molecular weight	930,000	155,000
Diffusibility	Poor	Good
Behavior relative to placenta	Held back by intact placenta	Passes through placenta readily
Half-life	Probably 2 wk. or less	30-35 days
Role in syphilis	Flocculating antibody	Complement fixing antibody
Role in disease	Precipitating and agglutinating antibody	Protective antibody; antitoxin
Role in allergy	Sensitizing antibody (reagin)	Blocking antibody
Role in erythroblastosis	Not significant	Major

*From Wiener, A. S., and Wexler, J. B.: An Rh-Hr syllabus, the types and their applications, ed. 2, New York, 1963. Grune & Stratton, Inc.

†The in vitro reactions are somewhat different, depending on the antigen-antibody system.

used by a conglutination method; if bivalent antibodies predominate, the saline agglutination test may be used. If antiserum containing a suitably balanced mixture of **Rh**$_0$ agglutinating and blocking antibodies is titrated in saline medium, a prozone (p. 1521) may occur.

Rh sensitization; Rh immunization The act of becoming sensitized to the Rh factor. This may occur in an Rh-negative individual as a result of a transfusion or an injection of Rh-positive blood or as the result of a pregnancy with an Rh-positive fetus. Injections of small doses (2 ml.) of Rh-positive blood into Rh-negative individuals, at 4 mo. intervals, cause more than 40% of subjects to become immunized (for preparation of anti-Rh serum). When injections are continued, more than 75% of Rh-negative persons can be immunized. Clinically, 2 widely spaced transfusions of Rh-positive blood into Rh-negative persons are more likely to lead to Rh sensitization than multiple transfusions in a short space of time. Fetal blood enters the maternal circulation during the course of approximately 1 out of 3 normal pregnancies and 1 out of 3 deliveries. Once a person has been sensitized to the Rh factor, he remains so for the remainder of his life, although the titer gradually becomes weaker. When this occurs, a small injection of Rh-positive blood will stimulate a pronounced and sustained rise in antibody titer.

Rh agglutination test A test to detect Rh antibodies of the bivalent type. When applied to sera from Rh-negative individuals being tested for Rh sensitization, a drop of the patient's serum is mixed with a drop of 2% saline suspension of the standard Rh-positive red cells (group O), and the reactions are read after the mixture has been incubated at body temperature for 1 hr. See Fig. 484.

Rh blocking test A test to detect Rh antibodies of the univalent variety. This test is also known as the indirect test for univalent Rh antibodies. The patient's undiluted serum or diluted serum is first mixed with a 2% saline suspension of Rh-positive red cells as in the agglutination test. If no clumping occurs, a drop of good anti-**Rh**$_0$ agglutinating serum of moderate titer is added and the mixture incubated 1 or 2 hr. at body temperature. See Fig. 484. The failure of clumping to occur despite the addition of anti-**Rh**$_0$ agglutinating serum or a distinct weakening of the reaction indicates the presence of **Rh**$_0$ blocking antibodies.

Rh conglutination test Another test for Rh antibodies of the univalent variety, also known as the direct test for univalent Rh antibodies. When applied to the patient's serum, the initial stage of the test is the same as that for the agglutination or blocking tests. After the red cells have sedimented, however, the supernatant fluid is removed as **completely as possible** and replaced by a drop of albumin-plasma mixture (3 or 4 parts pooled plasma and 1 part 30% bovine albumin). The cells are resuspended and the reactions read after another period of incubation. The technic can be carried out in a tube in a single step by using test cells suspended in 20% albumin solution. The conglutination test, when properly performed, is 5-40 times as sensitive as the blocking test for detecting univalent antibodies. Other modifications of the test have also been devised. See Fig. 485.

antiglobulin test, "developing" test, indirect Coombs test for Rh antibodies Another method for testing for univalent Rh antibodies. The test depends on the fact that the univalent antibodies coating the red cells are human serum globulin. A precipitating rabbit antiserum, prepared by injecting human serum or purified human serum globulin into rabbits (Coombs serum, commercially available), is first absorbed with pooled washed human red cells to remove heteroagglutinins and is then diluted to optimum strength. The patient's undiluted or diluted serum is allowed to react with a saline suspension of Rh-positive cells (group O). The sensitized cells are then washed 3 times

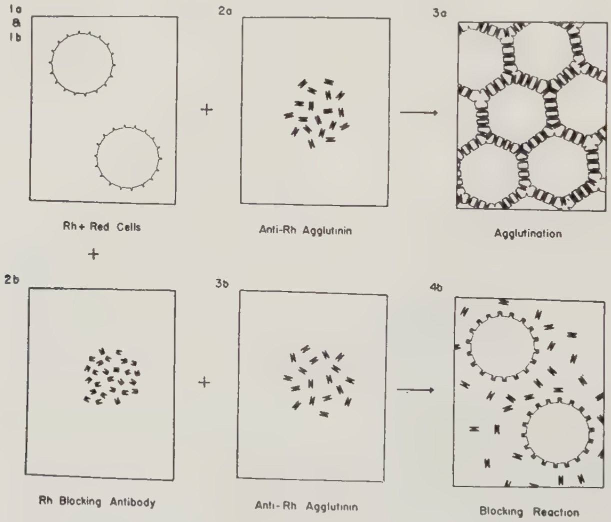

Fig. 484. Comparison of Rh agglutination and blocking reaction (test in saline media). (From Wiener, A. S.: Lab. Digest 14: No. 6, 1950.)

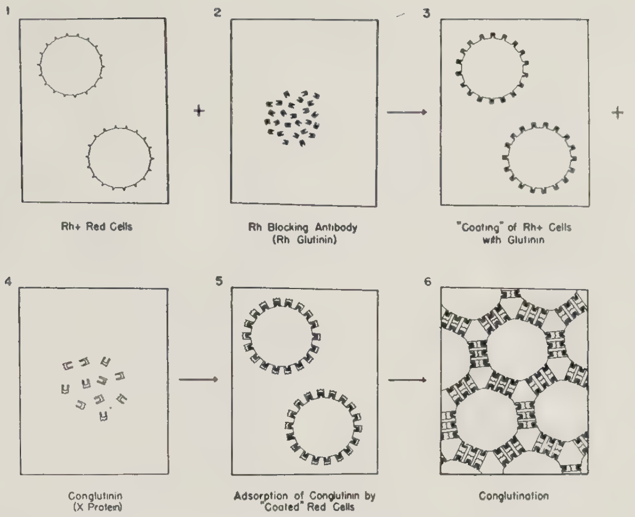
1
2
3
+
+
Rh+ Red Cells
Rh Blocking Antibody (Rh Glutinin)
"Coating" of Rh+ Cells with Glutinin
4
5
6
Conglutinin (X Protein)
Adsorption of Conglutinin by "Coated" Red Cells
Conglutination

Fig. 485. Rh conglutination reaction (test in plasma or serum media). (From Wiener, A. S.: Lab. Digest **14:** No. 6, 1950.)

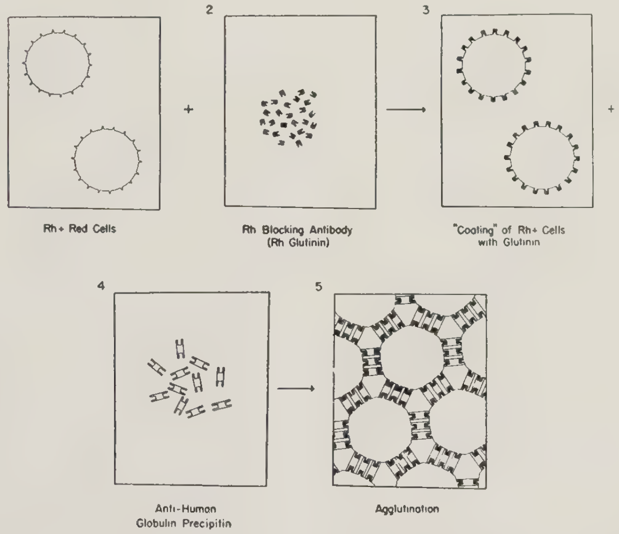
2
3
+
+
Rh+ Red Cells
Rh Blocking Antibody (Rh Glutinin)
"Coating" of Rh+ Cells with Glutinin
4
5
Anti-Human Globulin Precipitin
Agglutination

Fig. 486. Antiglobulin method of testing for univalent Rh antibodies. (From Wiener, A. S.: Lab. Digest **14:** No. 6, 1950.)

with a large volume of saline to remove the supernatant human serum proteins as completely as possible. A drop of Coombs serum is then added and the mixture centrifuged at 500 r.p.m. for 2 min. If the cells are then clumped, this indicates that they have been sensitized by univalent antibodies; failure to clump indicates the absence of sensitization. See Fig. 486.

Rh antibody tests with enzyme-treated cells If group O, Rh-positive red cells are washed 3 times to remove enzyme inhibitor and then treated with proteolytic enzymes such as trypsin or ficin, they become agglutinable by antisera containing the corresponding univalent Rh-Hr antibody. This test detects the same antibody as the blocking test, the conglutination test, and the antiglobulin test. The test with enzyme-treated cells equals the other tests in specificity and exceeds them in sensitivity. If the sensitivity of the blocking test can be rated as 1, then the sensitivity of the albumin-plasma conglutination test could be rated as approximately 30, the sensitivity of the antiglobulin test as approximately 50, whereas the sensitivity of tests made with enzyme-treated cells could be rated as approximately 100. The reactions obtainable in the tests with enzyme-treated cells are indicated diagrammatically in Fig. 487.

titration Determining the antibody concentration in an antiserum. Unlike chemical titrations, serologic titrations have a large intrinsic error, so that variations up to 100% or 200% are not unusual even in the hands of experts. In the usual technics the test cells are mixed with progressively doubled dilutions of the serum being examined in a series of tubes and the highest

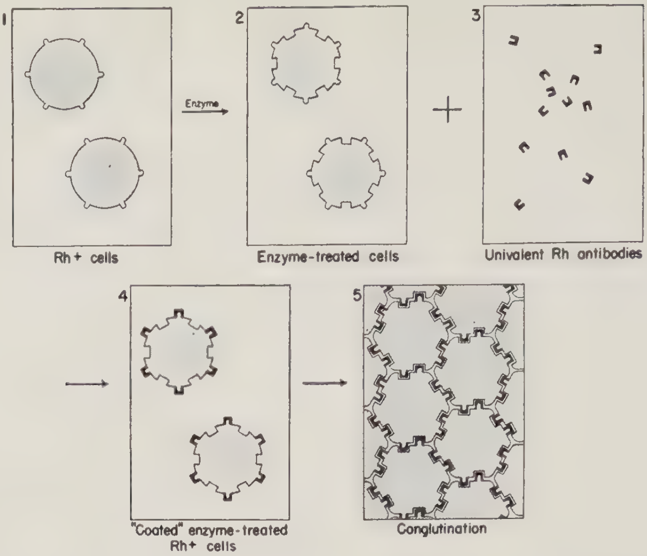

Fig. 487. Clumping of enzyme-treated Rh-positive cells by univalent Rh antibodies. (From Wiener, A. S.: Lab. Digest **14:** No. 6, 1950.)

dilution giving a distinct positive reaction determined.

titer of a serum The amount of specific antibody in an antiserum; the strength of a serum. This is usually expressed in units, which are best calculated by taking the reciprocal of the highest dilution giving a so-called 1-plus reaction. When determining the titer of a testing serum, the method of titration used should correspond to the method used in the actual tests, if the titer found is to have any practical value. The literature contains many reports of fantastically high titers, running into the millions, due in part to "carrying over" and in part to selecting vague reactions as the end point. To avoid such blunders, and in view of the intrinsic inaccuracy of the method, one should calculate the titer values by taking the average of many tests done on different days with many different test cells.

Rh$_0$ blocking serum An **Rh$_0$** antiserum containing univalent **Rh$_0$** antibodies of sufficiently high titer to give a distinct blocking reaction. Depending on the method used, an **Rh$_0$** antiserum must contain a minimum of 5-40 units of univalent antibody, as determined by the conglutination method, in order to give a distinct blocking reaction.

phenotypes The characteristics of individuals of a species as determined by direct observation, measurement, chemical or serologic testing, etc. Thus the characteristics, blue eyes, tall, short, harelip, hemophilic, group O, knock-knees, Rh$_1$-Rh$_2$, etc., all represent phenotypic characteristics. The phenotype may be partly or wholly genetically determined, or it may be entirely determined by the environmental influences, e.g., the color of the hair is usually genetically determined, but in some individuals it may merely be the result of beauty treatment.

Rh typing Classification of individuals within 1 of the 8 standard Rh types with the aid of anti-**Rh$_0$**, anti-**rh′**, and anti-**rh″** sera. Note the distinction between "Rh typing" and "Rh testing."

genotypes The constitutional make-up of an individual as determined by heredity. The genotype is transmitted through the germ cells by means of their chromosomes, although there is evidence also, in rare exceptional cases, of cytoplasmic inheritance. According to the gene theory each of the 23 pairs of chromosomes contains numerous genes arranged in linear order. Every individual has a pair of each kind of genes, one derived from the maternal parent and the other from the paternal parent. The make-up of the individual in terms of these genes is known as his "genotype."

Rh$_0$ phenotypes The 2 phenotypes, Rh positive and Rh negative, can be distinguished by using anti-**Rh$_0$** serum.

Rh$_0$ genotypes The 3 genotypes *RhRh*, *Rhrh*, and *rhrh* are based on Landsteiner and Wiener's theory that the **Rh$_0$** factor is inherited as a simple mendelian dominant by means of a pair of allelic genes, *Rh* and *rh*. Thus Rh-negative individuals are always homozygous *(rhrh)*. When 2 Rh-negative individuals marry, they produce only Rh-negative children. Rh-positive individuals may be either homozygous *(RhRh)* or heterozygous *(Rhrh)*.

homozygous Genetically pure, with genotype consisting of 2 identical genes. If an Rh-negative expectant mother is mated to a homozygous Rh-positive man, all the children will be Rh positive.

heterozygous Genetically impure, with genotype consisting of 2 different genes. If an Rh-negative expectant mother is mated to a heterozygous Rh-positive man, there is 50% chance that the children will be Rh positive and 50% chance that they will be Rh negative, or, as usually stated, 50% of the children will be Rh positive and 50% will be Rh negative.

Rh genes The series of allelic genes that determine the various sorts of Rh agglutinogens and Rh blood types (Table 313). Ten distinct varieties have been completely identified, namely, *r*, *r′*,

r'^w, r'', r^y, R^o, R^1, R^{1w}, R^2, and R^z. These genes each determine corresponding agglutinogens designated as rh, rh′, rh'^{w}, rh″, rh_y, Rh_o, Rh_1, Rh_1^w, Rh_2, and Rh_z. Note that in order to avoid ambiguity between symbols for genes and agglutinogens, italics are used and the letter "h" is dropped when designating genes. Also, while superscripts and subscripts are both used in the symbols for agglutinogens, only superscripts are used in the symbols for the genes in order to conform with the accepted practice of geneticists. Also note that, in addition to the shorthand symbols Rh_1 and Rh_2 (short for Rh_o' and Rh_o'', respectively), the symbol rh_y is used as an abbreviation for rh′″, while Rh_z is short for Rh_o'''. The most common of the 10 Rh genes among Caucasoids are *r*, R^1, and R^2, while R^o, r', and r'' are relatively rare, and r^y and R^z are extremely rare, so that the last 2 can usually be disregarded. On the other hand, R^o is the most common gene in Negroids, while R^z has its maximum frequency of about 3-5% among Mongoloids. *When discussing only the results of tests with* $\textbf{Rh}_\text{o}$ *serum,* which divides people into 2 types, Rh positive and Rh negative, only 1 pair of genes need be considered, namely, *Rh* and *rh*. Obviously, gene *Rh* comprises 5 genes, R^o, R^1, R^{1w}, R^2, and R^z while gene *rh* comprises 5 genes, *r*, r', r'^w, r'', and r^y (Wiener).

Some additional rare allelic genes Wiener has postulated the existence of additional rare alleles—r^v, r^G, R^{ov}, R^{oa}, R^{ob}, R^{oc}, R^{od}, $\mathfrak{R}^o$, $\bar{R}^o$, R^{1a}, R^{1b}, R^{1c}, R^{1d}, R^{1x}, R^{2x}, R^{2a}, R^{2b}, R^{2c}, R^{2d}, $\bar{\bar{R}}^o$, etc.

allelic genes; allelomorphic genes Alternate genes situated at corresponding loci in a pair of chromosomes. Thus genes that influence specific characters such as the blood group, eye color, etc., are located at a specific locus on a pair of chromosomes. All the alternate genes that can be located at a given locus are known as "allelic." Since the locus for the allelic genes of the A-B-O groups is on a different pair of chromosomes from the allelic genes of the M-N-S-U types, while the allelic genes of the Rh-Hr system are on a third pair of chromosomes, these 3 blood group systems are inherited independently of each other. (The Rh locus is not on the sex chromosomes.) When nonallelic genes for 2 distinct characteristics are on the same pairs of chromosomes, they are said to be **linked;** If they are on different chromosomes, they are said to be **independent.**

subtyping for $\textbf{rh}^\text{w}$ **factor** By using anti-$\textbf{rh}^\text{w}$ serum, a subdivision of the 8 Rh types can be made. Factor $\textbf{rh}^\text{w}$ occurs in approximately 5% of Caucasoids, and always in association with factor **rh**′. Thus type Rh_1 (Rh_o') is subdivided into Rh_1^w and Rh_1 proper, type Rh_z into $\text{Rh}_1^\text{w}\text{Rh}_2$ and Rh_1Rh_2 proper, and 1 or 2 examples of rare type rh'^w have been found. A similar subdivision of type rh_y is theoretically possible. The practical importance of the $\textbf{rh}^\text{w}$ factor is to explain rare puzzling examples of erythroblastosis fetalis when both mother and baby are type Rh_1, where the baby is subtype Rh_1^w and the mother has been sensitized to the $\textbf{rh}^\text{w}$ factor.

anti-Hr sera Antisera specific for the Hr factors. These are much rarer than anti-Rh sera, since the Hr factors are usually less antigenic than the Rh factors. Four anti-Hr sera are available: anti-**hr**′, anti-**hr**″, anti-$\textbf{hr}^\text{S}$, and anti-**hr**. The principal practical value of anti-**hr**′ serum is to subdivide type Rh_1 into the 2 types Rh_1Rh_1 and Rh_1rh, and the main value of anti-**hr**″ serum is to subdivide type Rh_2 into Rh_2Rh_2 and Rh_2rh. This gives a presumptive diagnosis of homozygosity or heterozygosity, and aids in anticipating the possible occurrence of erythroblastosis in the unborn child. Anti-**hr**″ sera are rarer than anti-**hr**′. Anti-$\textbf{Hr}_\text{o}$ serum has not been found.

Rh-Hr types, Rh-Hr phenotypes Types of human blood demonstrable by the combined use of Rh and Hr antisera. The number of phenotypes that can be distinguished depends on the number of antisera used in the tests; the evolution of our knowledge as more of the antisera became available is summarized in Table 314. When anti-**hr**′, anti-**hr**″, and anti-**hr** sera as well as anti-$\textbf{Rh}_\text{o}$, anti-**rh**′, and anti-**rh**″ sera are used, 28 Rh-Hr types can be distinguished corresponding to the 55 theoretically possible genotypes. Since $\textbf{Hr}_\text{o}$ antiserum and its presumptive antigen $\textbf{Hr}_\text{o}$ still have not been discovered, the examination will usually be confined to tests for **hr**′ and occasionally for **hr**″, in which case fewer phenotypes can be distinguished. It should be emphasized again that many of the phenotypes are quite rare, especially those involving the agglutinogens rh_y and Rh_z. In selecting the notations for the Rh-Hr types, the principle is employed that the first symbol shall represent the reactions with the Rh antisera, while the second symbol represents the reactions with the reciprocally related Hr antisera. For example, the phenotype Rh_2rh would represent blood reacting with Rh antisera anti-$\textbf{Rh}_\text{o}$ and anti-**rh**″ but not with anti-**rh**′ (Rh_2 = Rh_o + rh″) and with Hr antisera anti-**hr**′ and anti-**hr**″. The phenotype corresponding to any given genotype is determined by adding together the effects of the 2 genes that make up the genotype. For example, if it is desired to know the reactions given by the blood of an individual of genotype R^2r', proceed as in Table 312. The phenotype name for blood giving positive reactions with all 3 standard Rh sera and both standard Hr antisera is $\text{Rh}_\text{z}\text{rh}$, but since anti-$\textbf{Hr}_\text{o}$ serum to date exists only hypothetically,

Table 312. *Reactions of blood of* R^2r' *individuals*

		Reactions with Rh serum			*Reaction with Hr serum*		
		Anti-$\textbf{Rh}_\text{o}$	*Anti*-**rh**′	*Anti*-**rh**″	*Anti*-**hr**′	*Anti*-**hr**″	*Anti*-**hr**
Gene R^2	=	+	−	+	+	−	−
Gene r'.	=	−	+	−	−	+	−
Therefore, by addition, genotype R^2r'	=	+	+	+	+	+	−

the name Rh$_z$Rh$_o$ is used. If tests are made only with the 3 standard Rh antisera, the designation Rh$_z$ must be used.

Rh variants Some blood specimens give weak or intermediate reactions with one or more of the Rh-Hr antisera, demonstrating the existence of variants of the Rh-Hr factors. Of these, the most important are the variants of the **Rh**$_o$ factor, designated $\mathfrak{R}$h$_o$. $\mathfrak{R}$**h**$_o$ factor may occur in the absence of factors **rh**′ and **rh**″ (type $\mathfrak{R}$h$_o$), in association with **rh**′ but not **rh**″ (type $\mathfrak{R}$h$_1$), or in association with factor **rh**″ but not **rh**′ (type $\mathfrak{R}$h$_2$). **Rh**$_o$ variants are rare and occur far more frequently among Negroids than among Caucasoids. In Caucasoids as many as half of the specimens formerly typed as rh′ are now known to belong to type $\mathfrak{R}$h$_1$. The 3 agglutinogens $\mathfrak{R}$h$_o$, $\mathfrak{R}$h$_1$, and $\mathfrak{R}$h$_2$ are inherited by corresponding allelic genes $\mathfrak{R}^o$, $\mathfrak{R}^1$, and $\mathfrak{R}^2$.

Rh-Hr and C-D-E Use of the letters C, D, E for the Rh-Hr types is bound to lead to confusion and errors, since these symbols are based on a disproved genetic theory[62] and the misconception that blood factors and agglutinogens are identical. C = **rh**′; D = **Rh**$_o$; and E = **rh**″. Thus type rh is cde, type rh′ is Cde, type rh″ is cdE, type rh$_y$ is CdE, type Rh$_o$ is cDe, type Rh$_1$ is CDe, type Rh$_2$ is cDE, and type Rh$_z$ is CDE. Much confusion exists in the designation of the genotypes with this nomenclature, and it is suggested that it be dropped from the literature.

HISTORICAL

The Rh antigen was believed at first to be inherited on the basis of a single gene *Rh* and its allele *rh*. As already indicated, Wiener[62] then showed that there were actually at least 6 allelic genes that might possibly occupy a single locus in the chromosome. In 1941 Levine[63] described on the basis of an antibody in blood serum what was essentially just the opposite to Rh and called it Hr. The necessity of including this antigen within the sphere of the Rh antigen led to the possibility of another theory by Fisher and Race.[64] However, this theory had previously been disproved and discarded by Wiener.

In an editorial,[65] J. M. Hill discussed this entire question. He advised that in the light of present knowledge it seems best to abandon all concepts of dominance of Rh genes whether relative or absolute and simply consider all Rh types as determined by allelomorphic genes without dominance. Fisher's synthesis of the results of the British work on the isolation of Rh genes was briefly described by Race in 1944. The Fisher-Race theory proposes that there are 3 possible unlike genes that determine the Hr and Rh phenotypes of an individual. These genes are designated *C-c, D-d, E-e,* and the appropriate combinations of these result in the definite type of individual.

Coombs et al.[66] have discussed Fisher's views completely in an article entitled, "A new test for the detection of weak and incomplete Rh agglutinins." They stated that Fisher, noticing the mutually antithetical reactions of 2 of the 4 distinct types of sera then known (Race, Taylor, Cappell, and McFarlane, 1944) supposed that these 2 sera were reacting with allelic antigens. One of the antigens Fisher called C, and its allele c. The 2 remaining sera did not give antithetical reactions, so the antigens which they recognized were not allelic to each other though they presumably each had an allele. Instead of 7 alleles of the Rh gene at one locus as had previously been proposed, Fisher pictured 3 adjacent loci each with 2 alleles, each allele corresponding to an antigen that was capable of reacting with one antibody only, as shown diagrammatically in Table 315.

The hypothesis required 2 more antibodies, δ and η, unknown at that time. Mourant (1945) has found η. Thus Fisher predicted

Table 313. *Rh series of allelic genes*

Gene	Corresponding agglutinogen	*Reactions with Rh sera*				*Reactions with Hr sera*		
		*Anti-***Rh**$_o$	*Anti-***rh**′	*Anti-***rh**″	*Anti-***rh**^{w}	*Anti-***hr**′	*Anti-***hr**″	*Anti-***hr**
r	rh	−	−	−	−	+	+	+
r'	rh′	−	+	−	−	−	+	−
r'^{w}	rh′^{w}	−	+	−	+	−	+	−
r''	rh″	−	−	+	−	+	−	−
r^{y}	rh$_y$	−	+	+	−	−	−	−
R^{o}	Rh$_o$	+	−	−	−	+	+	+
R^{1}	Rh$_1$	+	+	−	−	−	+	−
R^{2}	Rh$_2$	+	−	+	−	+	−	−
R^{z}	Rh$_z$	+	+	+	−	−	−	−
R^{1w}	Rh$_1^w$	+	+	−	+	−	+	−
$\mathfrak{R}^{o}$	$\mathfrak{R}$h$_o$	Weak	−	−	−	+	+	+
$\mathfrak{R}^{1}$	$\mathfrak{R}$h$_1$	Weak	+	−	−	−	+	−
$\mathfrak{R}^{2}$	$\mathfrak{R}$h$_2$	Weak	−	+	−	+	−	−

Table 314. *Rh-Hr phenotypes and genotypes (international nomenclature)* (after Wiener)

2 Rh phenotypes			*12 Rh phenotypes*					*28 Rh-Hr phenotypes*					
Designations	*Approximate frequencies in N.Y.C. whites (%)*	*Reaction with anti-*$\mathbf{Rh}_o$ *(or anti-rhesus)*	*Designation†*	*Approximate frequencies in N.Y.C. whites (%)§*	*Reaction with*			*Designation*	*Approximate frequencies in N.Y.C. whites (%)§*	*Reaction with*			*55 Genotypes**
					*Anti-***rh′**	*Anti-***rh″**	*Anti-*$\mathbf{rh}^w$			*Anti-***hr′**	*Anti-***hr″**	*Anti-***hr**	
Rh negative	15	—	rh	14.4	–	–	–	rh	14.4	+	+	+	rr
			rh′	0.46‡	+	–	–	rh′rh	0.46	+	+	+	$r'r$
								rh′rh′	0.0036	–	+	–	$r'r'$
			rh'^{w}	0.004	+	–	+	$\mathrm{rh}'^{w}\mathrm{rh}$	0.004	+	+	+	$r'^{w}r$
								$\mathrm{rh}'^{w}\mathrm{rh}'$	0.00006	–	+	–	$r'^{w}r'$ or $r'^{w}r'^{w}$
			rh″	0.38	–	+	–	rh″rh	0.38	+	+	+	$r''r$
								rh″rh″	0.0025	+	–	–	$r''r''$
			rh_y	0.01	+	+	–	rh′rh″	0.006	+	+	–	$r'r''$
								$\mathrm{rh}_y\mathrm{rh}$	0.008	+	+	+	r^yr
								$\mathrm{rh}_y\mathrm{rh}'$	0.0001	–	+	–	r^yr'
								$\mathrm{rh}_y\mathrm{rh}''$	0.0001	+	–	–	r^yr''
								$\mathrm{rh}_y\mathrm{rh}_y$	0.000001	–	–	–	r^yr^y
			rh_y^w	0.00005	+	+	+	$\mathrm{rh}'^{w}\mathrm{rh}''$	0.00005	+	+	–	$r'^{w}r''$
								$\mathrm{rh}_y^w\mathrm{rh}'$	0.000001	–	+	–	$r'^{w}r^y$
Rh positive	85	+	Rh_o	2.8	–	–	–	Rh_o	2.8	+	+	+	R^oR^o or R^or
			Rh_1	50.7	+	–	–	$\mathrm{Rh}_1\mathrm{rh}$	33.4	+	+	+	R^1r, R^1R^o or R^or'
								$\mathrm{Rh}_1\mathrm{Rh}_1$	17.3	–	+	–	R^1R^1 or R^1r'
			Rh_1^w	3.3	+	–	+	$\mathrm{Rh}_1^w\mathrm{rh}$	1.6	+	+	+	$R^{1w}r$, $R^{1w}R^o$ or $R^or'^{w}$
								$\mathrm{Rh}_1^w\mathrm{Rh}_1$	1.7	–	+	–	$R^{1w}R^1$, $R^1r'^{w}$, $R^{1w}r'$, $R^{1w}R^{1w}$, or $R^{1w}r'^{w}$
			Rh_2	14.6	–	+	–	$\mathrm{Rh}_2\mathrm{rh}$	12.2	+	+	+	R^2r, R^2R^o or R^or''
								$\mathrm{Rh}_2\mathrm{Rh}_2$	2.4	+	–	–	R^2R^2 or R^2r''
			Rh_z	13.2	+	+	–	$\mathrm{Rh}_1\mathrm{Rh}_2$	13.5	+	+	–	R^1R^2, R^1r'' or R^2r'
								$\mathrm{Rh}_z\mathrm{rh}$	0.2	+	+	+	R^zr, R^zR^o or R^or^y
								$\mathrm{Rh}_z\mathrm{Rh}_1$	0.03	–	+	–	R^zR^1, R^zr' or R^1r^y
								$\mathrm{Rh}_z\mathrm{Rh}_2$	0.07	+	–	–	R^zR^2, R^zr'' or R^2r^y
								$\mathrm{Rh}_z\mathrm{Rh}_z$	0.0008	–	–	–	R^zR^z or R^zr^y
			Rh_z^w	0.6	+	+	+	$\mathrm{Rh}_z^w\mathrm{Rh}_1$	0.6	+	+	–	$R^{1w}R^2$, $R^{1w}r''$ or $R^2r'^{w}$
								$\mathrm{Rh}_1^w\mathrm{Rh}_2$	0.008	–	+	–	$R^{1w}r^z$, $R^{1w}r^y$ or $R^zr'^{w}$

*This table does not include hypothetical genes R^{zw} and r^{yw}, which, if they exist at all, are very rare.

†In this table $\mathbf{Rh}_1$ is used as a short designation for $\mathbf{Rh}_o'$, $\mathbf{Rh}_2$ is short for $\mathbf{Rh}_o''$, rh_y is short for rh′″, and $\mathbf{Rh}_z$ is short for $\mathbf{Rh}_o'''$.

‡The reduction in the frequency of type rh′ as compared with that given in earlier charts can be attributed to recognition of bloods of type $\mathfrak{R}\mathrm{h}_1$ (containing Rh_o variant) which are now included in type $\mathbf{Rh}_1$ instead of rh′. The agglutinogens $\mathfrak{R}\mathrm{h}_o$, $\mathfrak{R}\mathrm{h}_1$ and $\mathfrak{R}\mathrm{h}_2$ and their corresponding genes $\mathfrak{R}^o$, $\mathfrak{R}^1$ and $\mathfrak{R}^2$ are not given here because this would serve unnecessarily to complicate the chart by increasing the number of possible genotypes to 91. Also, no attempt is made to include certain rare exceptional bloods, such as those lacking both factors **rh′** and **hr′** or lacking both **rh″** and **hr″**, etc.

§Based on the estimated gene frequencies, $r = 0.38$, $r' = 0.006$, $r'' = 0.005$, $r^y = 0.0001$, $r'^{w} = 0.00005$, $R^o = 0.027$, $R^1 = 0.41$, $R^2 = 0.15$, $R^z = 0.002$, and $R^{1w} = 0.02$.

the existence of 3 antisera reciprocally related to the 3 principal anti-Rh sera. At the time this prediction was made, one of these antisera, anti-**hr′** (or anti-c), was already known. Of the remaining 2, only 1, anti-**hr″** (or anti-e) has been shown with certainty to exist. Instead of anti-$\mathbf{Hr}_0$ (anti-d), a different antiserum, anti-**hr** (or anti-f), has been found, which was not predicted by Fisher.

It may be convenient at this point to summarize the contributions made by British investigators to the field of blood grouping during the past decades. This was initiated in 1942 by Taylor and Race, following publication of the American investigations on the Rh factor. After Taylor's premature death Race took over the leadership of British work and soon confirmed and extended American reports on the serology and heredity of the Rh-Hr blood types. By studying difficult problems of multiple sensitization he and his collaborators discovered the blood factors $\mathbf{rh}^w$, **Levay,** and **Lutheran.** These and other contributions of British investigators are well summarized in the book of Race and Sanger, the third edition of which has been published.[67] Mourant, co-discoverer of the **Lewis** factor, has published an important book[68] summarizing advances in anthropologic aspects of the subject. Mollison, who with Cutbush and Parker discovered the **Duffy** (**F**) factor, studied exchange transfusion and published a fine book on blood transfusion,[69] while Pickles contributed a book on erythroblastosis fetalis.[70] Finally, Dacie has specialized in the study of autosensitization and has published a book on hemolytic anemia.[71] Mention should also be made of the contributions of Coombs and Mourant to the detection of univalent antibodies.[71a]

Table 315

Antibodies	*Loci on chromosome*	*Antibodies*
Γ	C or c	γ
Δ	D or d	δ
Η	E or e	η

Table 316. *Rh antibodies and agglutinogens*

Original names Fisher's antigenic or genetic notation Antibodies			*Agglutinogens**							
Wiener	*Fisher*	*Cappell*	Rh_1 *CDe*	Rh_2 *cDE*	*rh* *cde*	Rh_0 *cDe*	*rh′* *Cde*	*rh″* *cdE*	Rh_z *CDE*	rh_y *CdE*
Anti-**rh′**	Γ	Anti-C	+	−	−	−	+	−	+	+
Anti-$\mathbf{Rh}_0$	Δ	Anti-D	+	+	−	+	−	−	+	−
Anti-**rh″**	Η	Anti-E	−	+	−	−	−	+	+	+
Anti-**hr′**	γ	Anti-c	−	+	+	+	−	+	−	−
Anti-$\mathbf{Hr}_0$	δ	Anti-d	(−)	(−)	(+)	(−)	(+)	(+)	(−)	(+)
Anti-**hr″**	η	Anti-e	+	−	+	+	+	−	−	−
Anti-**hr**	None†	None†	−	−	+	+	−	−	−	−

*One must not confuse the agglutinogens with the types. The types (phenotypes) being determined by a pair of genes (genotypes) therefore necessarily comprise a pair of agglutinogens.

†No name given by Fisher or Cappell; anti-f used by Rosenfield et al.

Rh-Hr NOMENCLATURE AND THE C-D-E NOTATIONS

Wiener and Wexler[72] have summarized the fundamental differences between the Rh-Hr nomenclature and the C-D-E notations.

1. Implicit in the C-D-E notations is the concept that each Rh-Hr agglutinogen has but a single blood factor with a single corresponding antibody, making it unnecessary to distinguish between blood factor and agglutinogen. For example, instead of the agglutinogen Rh_1, a set of 4 agglutinogens, *C, D, e, F,* is visualized. As has been pointed out, however, a fundamental principle in immunology is that antigens have multiple corresponding antibodies and thus are characterized by multiple serologic factors. This applies not only to the agglutinogens of the human blood groups but also to the blood groups of cattle, fowl, mice, and even to the antigens of bacteria and viruses, and soluble proteins. The Rh-Hr nomenclature with its distinctive type faces for agglutinogen and blood factors automatically allows for this fundamental serologic reality.

2. The C-D-E-F notations are based on the concept of inheritance of the agglutinogens in uniform sets of 3 (or 4 if postulated F-f pair is included). Actually the number of serologic factors that characterize an agglutinogen is unlimited, because of the complex character of the reacting sites, and thus varies from agglutinogen to agglutinogen, as studies on the Rh-Hr agglutinogens have shown. In this respect the Rh-Hr nomenclature is realistic.

3. The Rh-Hr terminology takes into ac-

count the existence not only of sharply differentiated blood factors but also of graded series of related factors, the latter being indicated by varying the type face, as in the case of $\mathbf{Rh_0}$ variants. The C-D-E notations treat the 2 cases in the same manner. As a result, while the blood factors $\mathbf{C^w}$, $\mathbf{C^x}$ have their corresponding specific antisera anti-$\mathbf{C^w}$, anti-$\mathbf{C^x}$, certain variants of $\mathbf{Rh_0}$ are indicated by the analogous symbol D^u even though there is no corresponding anti-D^u serum.

4. A logical sequel of the concept of a one-to-one correspondence between antigen and antibody is the interpretation of certain sera as containing "linked" antibodies. For example, in the case of the A-B-O system, the anti-**C** antibody had to be designated anti-$A + B$ even though the more reasonable interpretation of the reaction of such antiserum is that it identifies an additional serologic factor of red cells, namely, blood factor **C**. Similarly, in the Rh-Hr system linked antibodies anti-$C + C^w$ had to be postulated to explain the reactions of anti-**rh′** serum with bloods Rh_1^w (CD^weF) as well as Rh_1 (DCeF).

5. The C-D-E notations pretend to indicate in the symbols all of the reactions of the red cell and are therefore presumed to be self-explanatory. The Rh-Hr nomenclature makes no attempt to include all the serologic attributes of the agglutinogen in the symbols, which instead are in the nature of simple mnemonics, as has already been explained. Of interest are the contrasting effects on the symbols of the discovery of anti-*f* (anti-**hr**) and anti-*V* (anti-$\mathbf{hr^v}$) sera. Although the Rh-Hr symbol of gene *r* remained essentially unchanged, the corresponding C-D-E symbol *cde* had to be expanded first to *cdef* and then to *cdefV*. Moreover, it has proved impossible to expand the C-D-E notations to keep abreast of the newer discoveries, such as $\mathbf{Rh^A}$, $\mathbf{Rh^B}$, $\mathbf{Rh^C}$, $\mathbf{Rh^D}$, etc. In fact, in some instances in which the discoveries have not followed the theoretical predictions, symbols for certain factors have been included in the notations even though the factors have not yet been proved to exist, as in the case of *d* and *F*.

6. A serious difficulty with the C-D-E-F notations is the lack of agreement in the use of these symbols by different workers. This is particularly true of the symbols for the various phenotypes. For example, the phenotype Rh_1rh has been variously designated as CcDee; CDe/cde; (+++−); (++−+); C+c+D+E−e+; DCe/−ce; DCeF/dcef; C(A+B+), D(A+), E(A-B+), F(A+); etc.

7. Instead of reporting phenotypes, many workers who use the C-D-E symbols report "most likely genotypes." In fact, the phrase "most likely" is frequently omitted and "genotypes" are reported based on serologic tests alone. This procedure introduces significant statistical errors and in a number of instances has resulted in confusion. As has been pointed out, the Rh-Hr nomenclature has distinctive symbols for phenotypes and genotypes, which automatically avoid this pitfall.

The Committee on Medicolegal Problems of the American Medical Association prepared a report[73, 74] on the medicolegal application of blood grouping tests in which one of the most important problems discussed was that of Rh-Hr nomenclature. In the report the recommendation was made that "unless and until some other convention can be agreed upon, the original Rh-Hr notations be retained as the standard and sole nomenclature for the preparation of approved medicolegal reports on Rh types." In view of the facts that are presented here, it would appear that this recommendation should now be extended to include the applications of the Rh-Hr blood types in clinical medicine and anthropology.

As Wiener has shown, the essential difference between the 2 concepts is on a serologic and not a genetic level. Blood grouping is a subspecialty of immunology, and although a knowledge of genetics is helpful, it is only of secondary importance for mastery of the field. The linkage theory completely evades the fundamental immunologic concept of the difference between an agglutinogen and its serologic attributes, the blood factors, and therein lie the pitfalls of the C-D-E notations. The issue is thus not merely a choice between 2 nomenclatures but also the accuracy in reporting scientific observation.

Rh FACTOR

Reports of posttransfusion reactions when bloods of homologous groups have been transfused, where preliminary tests have been made by experienced observers using reliable and potent sera, indicate that these reactions represent intragroup incompatibilities. Cases of this type have been observed with unusual frequency following primary transfusions given to pregnant or postpartum women. These represent an example of isoimmunization.

It was formerly believed that dangerous transfusion reactions could not occur if both donor and recipient belonged to the same

Landsteiner blood group. Between 1921 and 1939, with the increasing use of blood transfusion, authenticated reports of hemolytic transfusion reactions began to appear in the literature. These hemolytic reactions occurred in spite of the fact that all blood grouping tests showed donor and recipient of the same group and apparently compatible. Unger[56] had observed the first cases of intragroup incompatibility. Both patient and donor belonged to group O, but on cross matching some clumping of the cells was observed. When such blood was transfused, the patient developed chills and fever. Unger believed this reaction was hemolytic in nature. He postulated the existence of 2 different antibodies, the anti-**A** and anti-**B**, which he called the "major" agglutinins, and another antibody which he called the "minor" agglutinin. In cross matching he used cells suspended in serum rather than in saline; he therefore inadvertently, even at that early date, was carrying out the conglutination test. Possibly he was detecting the $\mathbf{Rh}_0$ univalent antibody, although he did not identify it.

In most of the subsequently reported cases no explanation of reactions could be found. In a few instances the patient's blood serum was found to contain **irregular isoagglutinins** and **isohemolysins.** The irregular isoantibodies acted not only on the blood of the donor used in the transfusion but on certain other human bloods as well, independently of the blood groups. Among the most carefully studied instances of this sort were those reported by Unger,[56] Zacho,[75] Culbertson and Ratcliffe,[76] Neter,[77] and Levine and Stetson.[78] No attempt, however, was made to correlate these cases, and the subject remained in this stage until Landsteiner and Wiener reported their discovery of the Rh factor and explained its role in reactions in retransfusions.

Schiff had found that injection of sheep blood into rabbits caused formation of anti-**A** agglutinins and hemolysins. When Landsteiner and Wiener in 1937 detected the presence of M-like agglutinogens in the blood of rhesus monkeys, they found that injection of rhesus monkey blood into rabbits stimulated production of anti-**M** agglutinins. They continued their research in the hope that by using animal blood instead of human blood for preparing antisera they might discover new factors in human blood. They did find that some of their antisera against rhesus monkey blood contained an agglutinin reacting also with the blood cells of 85% of the Caucasoid population, quite independently of the 4 blood groups O, A, B, and AB, or **M**, **N**, and **P** factors. They designated the agglutinogen detected by their anti-rhesus serum "Rh" to indicate the manner in which it was discovered,[79] using the first two letters of the word "rhesus." They did not immediately report their findings because they wished to perform other work on their antisera. In 1939 Wiener and Peters found 3 instances of hemolysis following transfusions of blood of the homologous groups, and these were proved to be due to isoimmunization to the rhesus factor. Thus this new blood factor became clinically very important,[80] and the cases were reported. Wiener stated in his Alvarenga Prize Lecture[81] that this was the reason why the Rh factor found by Landsteiner and Wiener in 1937 was first announced in January, 1940.

Briefly, the blood cells of 85% of all Caucasoids are agglutinated by anti-rhesus (anti-$\mathbf{Rh}_0$) sera. Such cells contain the Rh agglutinogen identified by the factor now designated as $\mathbf{Rh}_0$. These individuals were called Rh positive, whereas the remaining 15%, whose cells lacked the factor, were called Rh negative. Since these Rh-negative blood cells do not possess the $\mathbf{Rh}_0$ factor they therefore are not agglutinated by anti-$\mathbf{Rh}_0$-containing sera.

Since 1940 other Rh factors have been found, **rh′**, **rh″**, $\mathbf{rh}^w$, the variant $\mathfrak{R}\mathbf{h}_0$, and many others, which are described later. Unfortunately, a so-called "simplified nomenclature" was introduced into the literature, following the Fisher "synthesis," but since the nomenclature is based on an erroneous premise, it should be eliminated from the literature. It is neither simple nor correct, as explained on pp. 1496 and 1497.

In recent years attention has been called to the fact that agglutinogens have certain serologic attributes, which Wiener has called "factors"; they are the characteristics by which the agglutinogens are identified. Thus different antisera will cause clumping of cells containing their specific factors, but this does not indicate that all agglutinogens containing such factors are alike. For example, agglutinogen A has a blood factor **C** that it shares with agglutinogen B and another factor $\mathbf{F}_A$ that it shares with sheep red cells.

Of all the blood systems the Rh-Hr is the most complex and, except for the A-B-O system, the most important. The original rhesus factor found by Landsteiner and Wiener and designated $\mathbf{Rh}_0$ is spelled with

Table 317. *Blood groups in man: role in blood transfusion**

System	*Partial list of blood factors*	*Some identifiable types*	*Isoantibodies*		*Role in causing transfusion hemolysis*
			Natural	*Immune*	
A-B-O	**A**, **B**, **C**, **O**, and variants such as A_1, A_2, $A_{1,2}$ and A_3, etc	6 major groups and subgroups—O, A_1, A_2, B, A_1B, A_2B; also rarer subgroups A_3, A_3B, etc.	Regularly present except during neonatal period	Common	Most important
M-N-S-U	**M**, **N**, **M**^{e}, **S**, **s**, **Hu** (Hunter), **He** (Henshaw), **U**, and variants such as M_2, N_2, etc.	6 major types using more readily available sera and anti-**M**, anti-**N**, and anti-**S**	Rare	Rare	Has caused hemolysis in rare cases
Rh-Hr	**Rh**$_0$, **Rh**^{A}, **Rh**^{B}, **Rh**^{C}, **Rh**^{D}, **rh′**, **rh″**, **rh**^{w}, **hr′**, **hr″**, **hr**, **hr**^{S}, **Hr**, and variants such as $\mathfrak{R}$**h**$_0$	28 major types, but of these only 9 with frequencies greater than 1%	A few questionable cases reported	Common	Most frequent cause of intragroup transfusion hemolysis
P-Tj^a	**P** and variants Tj^a	2 major types	Infrequent	Very rare	No cases reported
K-k	**K** and **k**, **Kp**^{a},**Kp**^{b}	3 types	None reported	Occasional	Rare cause of hemolysis in isosensitized patients
Lutheran	**Lu**^{a}, **Lu**^{b}	3 types	None reported	1 case	Very rare cause of hemolysis in isosensitized patients
Duffy (Fy) F-f	**F** and **f**	2 major types, because anti-**f** serum not available	None reported	Several cases	Rare cause of hemolysis
Lewis (Le)	**Le**^{a} and **Le**^{b}	3 types (?)	Infrequent	None found	Plays no role in transfusion hemolysis; **Le**^{a} factor reciprocally related to secretor trait
Kidd (Jk)	**J** and **j**	2 major types, because anti-**j** serum not available	None reported	Few cases	Rare cause of hemolysis
"Private" blood types†					
Rare blood factors	**Levay**, **Gr** (Graydon), **Mi**^{a} (Miltenberger), Becker, **Be**^{a} (Berrens), **Ca**§ (Cavaliere)	Positively reacting bloods very rare	None reported	Very rare	Very rare cause of hemolysis
Factors with almost universal distribution‡	**Tj**^{a}, **U**, **Vel**, **Ge**	Negatively reacting bloods very rare	None reported	1 case anti-**U**; 8 cases anti-**Tj**^{a}	Very rare cause of transfusion hemolysis

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*Modified after Wiener, A. S.: New York J. Med. **54**:3071, 1954.

†In the case of the rare blood factors, other individuals possessing such factors are most easily found by testing relatives of the proband, the person in whose blood the factor was first found. Similarly, individuals lacking factors of almost universal distribution are to be found most readily among relatives of the proband. The hereditary nature of the blood factors, in general, is most noticeable in the case of very rare blood types, and Levine has therefore designated them as "private" blood types to indicate their restriction to certain rare families.

‡Factor **k** of the K-k blood types could also be included under this classification.

§Ca and Wr^a are the same—Ca is the preferred symbol.

a capital R to show its great antigenicity and clinical importance. When additional Rh factors were discovered and their relationship to **Rh$_o$** established, the small letter r was used since these were not as antigenic as the originally found rhesus factor **Rh$_o$**. The 2 principal factors aside from **Rh$_o$** are **rh′** and **rh″**. These 3 factors determine the 8 principal Rh types since they may occur alone or in combination.

Eight principal Rh types

When these 3 factors occur alone, they determine 3 Rh types as follows: Rh$_o$, rh′, and rh″.

In combination, the following types may be present in blood:

Rh$_1$, originally called Rh$_o$′, contains **Rh$_o$** and **rh′**.

Rh$_2$, originally called Rh$_o$″, contains **Rh$_o$** and **rh″**.

Rh$_z$, originally called Rh$_o$′″, or Rh$_1$Rh$_2$, contains all 3 factors, **Rh$_o$**, **rh′**, and **rh″**.

rh$_y$, originally called rh′rh″, contains **rh′** and **rh″**.

rh, also called triple Rh negative, contains none of the 3 principal factors.

Table 318. *"Standard" Rh types and percentages*

Rh type	*% in each "standard" type*	*Rh type*	*% in each "standard" type*
rh	14.4	Rh$_o$	2.1
rh′	0.46	Rh$_1$	50.7
rh$'^{W}$	0.004	Rh$_1^{W}$	3.3
rh″	0.38	Rh$_2$	14.6
rh$_y$	0.014	Rh$_z$	13.4
rh$_y^{W}$	0.000051	Rh$_z^{W}$	0.6

Table 319. *List of known Rh-Hr antibodies and the factors they detect**

Antibodies	*Blood factors*
1. Anti-rhesus and anti-**Rh$_o$**	**Rh$_o$** and **$\mathfrak{R}$h$_o$**
2. Anti-**rh′**	**rh′**
3. Anti-**hr′**	**hr′**
4. Anti-**rh″**	**rh″**
5. Anti-**hr″**	**hr″**
6. Anti-**rh**^{w1}	**rh**^{W1}
7. Anti-**hr**	**hr**
8. Anti-**rh**^{x1}	**rh**^{x1}
9. Anti-**hr**^{V}	**hr**^{V}
10. Anti-**rh**^{W2}	**rh**^{W2}
11. Anti-**rh**^{G}	**rh**^{G}
12. Anti-**rh**$_i$	**rh**$_i$
13. Anti-**Rh**^{A}	**Rh**^{A}
14. Anti-**Rh**^{B}	**Rh**^{B}
15. Anti-**Rh**^{C}	**Rh**^{C}
16. Anti-**Rh**^{D}	**Rh**^{D}
17. Anti-**Hr**	**Hr**
18. Anti-**hr**^{S}	**hr**^{S}

*From Wiener, A. S.: J. Forensic Med. 7:93, 1960.

The blood factors are arranged chronologically in the order of their discovery.

The numbers are not intended as a basis for a new nomenclature but may be used as a key if desired.

Variants of Rh$_o$ factor

(1) **Variant $\mathfrak{R}$h$_o$:** In addition to the principal Rh types, a variant of the **Rh$_o$** factor, called **$\mathfrak{R}$h$_o$**, was found by Wiener in 1944.[83] He showed that certain blood specimens gave weak but distinct reactions with certain Rh antisera and appeared to occupy a position intermediate between negatively reacting bloods and those that gave sharp positive reactions. Family studies showed this characteristic to be hereditary. These types are at times designated as **intermediate,** and the genes that determine them are called **intermediate genes.** Although there are other Rh variants, the variant of **Rh$_o$** seems to be the most important because of its antigenicity. The **$\mathfrak{R}$h$_o$** factor, alone or in combination with the 2 Rh factors **rh′** and **rh″**, determines 4 other Rh types as follows:

$\mathfrak{R}$h$_o$, with only **$\mathfrak{R}$h$_o$**
$\mathfrak{R}$h$_1$, with **$\mathfrak{R}$h$_o$** and **rh′**
$\mathfrak{R}$h$_2$, with **$\mathfrak{R}$h$_o$** and **rh″**
$\mathfrak{R}$h$_z$, with **$\mathfrak{R}$h$_o$**, **rh′**, and **rh″**

Unger and Wiener have described variants of **Rh^{A}**, **Rh^{B}**, **Rh^{C}**, and **Rh^{D}** (p. 1502). To distinguish these, the Greek alphabet is used; for example, **Rh$^{\alpha}$**, **Rh$^{\beta}$**, **Rh$^{\delta}$**, **Rh$^{\gamma}$**. Approximately 50% of $\mathfrak{R}$h$_o$ bloods lack one or more of the associated factors **Rh^{A}**, **Rh^{B}**, **Rh^{C}**, and **Rh^{D}**. A superscript small letter is used to designate this, for example, $\mathfrak{R}$h$_1^{a}$rh, $\mathfrak{R}$h$_1^{ad}$rh, etc.

Factor **$\mathfrak{R}$h$_o$** comprises a group giving reactions of intermediate intensity with anti-**Rh$_o$** sera. Blood specimens that react typically with most anti-**Rh$_o$** sera but give slightly weaker reactions with a number of sera are known as high-grade variants, whereas those that react consistently weakly with all anti-**Rh$_o$** sera are low-grade variants. The reactions are determined by the following characteristics: in saline media they fail to react with anti-**Rh$_o$** sera, even those that contain potent anti-**Rh$_o$** agglutinins. They frequently but not always react with univalent **Rh$_o$** antisera by the conglutination method. They almost always react with such sera by the antiglobulin technics, although the avidity and titer of the reactions are generally lower than those for typical **Rh$_o$**-positive bloods. Wiener[84] has pointed out that the bloods of chimpanzees give reactions resembling those of high-grade Rh$_o$ variants.

Table 320. *Reactions of blood with Rh antisera*

*Anti-***rh′**	*Anti-***rh″**	*Anti-*$\mathbf{Rh}_0$	*Phenotype*	*Principal Rh factors present*
Blood lacking $\mathbf{Rh}_0$				
–	–	–	rh, or triple Rh negative	None
+	–	–	rh′	**rh′**
–	+	–	rh″	**rh″**
+	+	–	rh$_y$	**rh′, rh″**
Blood containing $\mathbf{Rh}_0$				
–	–	+	Rh$_0$	$\mathbf{Rh}_0$
+	–	+	Rh$_1$	$\mathbf{Rh}_0$**, rh′**
–	+	+	Rh$_2$	$\mathbf{Rh}_0$**, rh″**
+	+	+	Rh$_z$	$\mathbf{Rh}_0$**, rh′, rh″**

$\mathfrak{R}h_0$ is relatively rare among Caucasoids; it is more frequently found among Negroids.

The important fact to remember is that if this Rh$_0$ variant remains undetermined by laboratory tests, an individual of type $\mathfrak{R}h_1$ will be erroneously typed as rh′, whereas an $\mathfrak{R}h_2$ person will be mistakenly identified as rh″. Those belonging to $\mathfrak{R}h_z$ could be mistakenly identified as rh$_y$. If blood of such individuals is transfused into patients lacking $\mathfrak{R}h_0$, the patient might develop anti-$\mathbf{Rh}_0$ agglutinins and be sensitized. Unfortunately these anti-$\mathbf{Rh}_0$ agglutinins react not only with $\mathfrak{R}h_0$ but with any blood containing the $\mathbf{Rh}_0$ factor as well.

(2) **Type** $\bar{\bar{\mathbf{R}}}\mathbf{h}_0$**:** In 1951, Race et al.[85] found in the serum of a mother of an erythroblastotic baby an antibody that clumped 1400 consecutive blood specimens but failed to clump the mother's own cells. Her red cells reacted with anti-$\mathbf{Rh}_0$ serum but appeared to lack contrasting factors of the 2 pairs **rh′-hr′** and **rh″-hr″** (see under genotypes of the Rh-Hr system), and that is why a double bar is placed above the R in the symbol for this type. These investigators postulated that a deletion had occurred in the Rh "chromosomes" and asserted that the genotype of the mother was -D-/-D-. Wiener disagreed with this interpretation and presented his views based on serologic and genetic principles.

The red cells of this patient were agglutinated in saline media with anti-$\mathbf{Rh}_0$ blocking serum which failed to clump ordinary $\mathbf{Rh}_0$-positive cells under identical conditions. This individual therefore had an agglutinogen similar to agglutinogen Rh$_0$ but differing in its higher reactivity with anti-$\mathbf{Rh}_0$ serum. This property is the fundamental characteristic of the agglutinogen and is due to a modification in its chemical structure. This modification results in increased reactivity with anti-$\mathbf{Rh}_0$ sera and simultaneously crowds out the site of reactivity with all other anti-Rh and anti-Hr sera. For this new type of antigen, Wiener proposed the term **super** $\mathbf{Rh}_0$ and originally assigned the symbol "bar" Rh$_0$, written $\bar{\mathrm{R}}\mathrm{h}_0$. To avoid confusion with another, related type (see below), this symbol has now been changed to double bar Rh$_0$, written $\bar{\bar{\mathbf{R}}}\mathbf{h}_0$.

In an unpublished personal communication (1962) Dr. Unger described a type $\bar{\bar{\mathrm{R}}}\mathrm{h}_0$ blood found in his laboratory after deliberately searching for such a type for approximately 10 yr. He has tested many thousands of bloods during that period, and this is the first one that he has found, pointing to the fact that such blood is very rare. This is a Puerto Rican woman, 82 yr. of age, who required a hysterectomy. She was anemic and a blood transfusion was ordered. All bloods tested were incompatible. The patient's blood was then referred to Dr. Unger. Examination showed the blood to be group O, type MN, type $\bar{\bar{\mathrm{R}}}\mathrm{h}_0$. Unger routinely tests the serum of every donor and every patient for atypical antibodies, using the sensitization test. He found that the serum of this patient contained an antibody. The titer was high and the antibody clumped the cells of all donors' bloods but not her own cells. On further investigation the antibody was classified as anti-**Hr.** (Anti-**Hr** is produced by sensitized persons homozygous for the so-called double bar genes, $\bar{\bar{\mathrm{R}}}\mathrm{h}_0$, $\bar{\bar{\mathrm{R}}}\mathrm{h}^{\mathrm{w}}$, etc.) The bloods of members of her family are being examined, but this has not been completed. The bloods of her children are available, but obviously they are heterozygous for this rare gene and add nothing of interest. Her siblings are in Puerto Rico. At the present writing Unger is making arrangements to obtain the bloods of her siblings in order to complete this study.

Since this variant crowds out the site of reactivity with all other Rh-Hr antisera, the existence of the factor with its gene can give rise to contradictions to the rule that an **rh′**-negative person cannot be the parent of an

hr′-negative child and that an **rh″**-negative person cannot be the parent of an **hr″**-negative child. This subject is considered in medicolegal applications and will be discussed later.

(3) **Type $\bar{\text{R}}\text{h}_0$:** The single bar Rh_0 refers to those individuals whose Rh_0 agglutinogen is characterized by absence of the single pair of contrasting blood factors **rh″** and **hr″**. This type was first described by Wiener and associates.

Other Rh_0 factors

(4) **Type $\bar{\bar{\text{R}}}\text{h}^{w\prime}$:** Refer to $\bar{\bar{\text{R}}}\text{h}_0$ and rh^{w}.

(5) **Rh**$^{\text{A}}$, **Rh**$^{\text{B}}$, **Rh**$^{\text{C}}$, **Rh**$^{\text{D}}$ antibodies related to anti-**Rh**$_0$ have been found by Wiener, Unger, and their associates, in the plasma of persons who have what appear to be typical Rh agglutinogens or variants of the **Rh**$_0$ factor (types Rh_0, Rh_1, Rh_2, and Rh_z and their variants). These individuals usually attract attention because of a hemolytic transfusion reaction or by giving birth to an erythroblastotic baby, after which an antibody is detected in this Rh-positive patient's serum. The antibody seems at first to be identical in specificity to anti-**Rh**$_0$ but differs from typical anti-**Rh**$_0$ in that it does not react with the patient's own Rh-positive blood cells or with those of certain other **Rh**$_0$-positive persons.

Four such antibodies have already been discovered: These are called anti-**Rh**$^{\text{A}}$, anti-**Rh**$^{\text{B}}$, anti-**Rh**$^{\text{C}}$, and anti-**Rh**$^{\text{D}}$.[86-91] The 4 blood factors that they detect may occur in an Rh agglutinogen in any of 16 theoretically possible combinations. **Standard** Rh-positive blood has all these factors, **Rh**$^{\text{A}}$, **Rh**$^{\text{B}}$, **Rh**$^{\text{C}}$, and **Rh**$^{\text{D}}$, as well as **Rh**$_0$. Red cells giving any of the **other theoretically possible reactions** are found in only about 1.6% of Rh-positive Negroes and in only about 0.5% of Rh-positive Caucasians.

Wiener and Unger[92] have explained the foregoing by using the outline of the human hand as a diagrammatic device for representing the Rh_0 portion of the Rh agglutinogens. The webs at the bases of the 4 interspaces between the fingers may be considered to be combining sites for the attachment of antibodies. The web between the thumb and index finger may be considered the site of the combining group for anti-**Rh**$_0$, that between the fourth and fifth fingers for anti-**Rh**$^{\text{A}}$, the web between the third and index fingers for anti-**Rh**$^{\text{C}}$, etc. All so-called "standard" Rh agglutinogens must have the combining site for anti-**Rh**$_0$, but agglutinogens might lack the combining sites for one or more of the antibodies anti-**Rh**$^{\text{A}}$, anti-**Rh**$^{\text{B}}$, anti-**Rh**$^{\text{C}}$, anti-**Rh**$^{\text{D}}$, etc. Since almost all Rh-positive bloods have all associated blood factors **Rh**$^{\text{A}}$, **Rh**$^{\text{B}}$, **Rh**$^{\text{C}}$, **Rh**$^{\text{D}}$, etc., as well as **Rh**$_0$, special complex symbols are assigned **only for the exceptional bloods that lack one or more of the primary blood factors.**

Individuals who lack one of these factors, for example, **Rh**$^{\text{A}}$ (agglutinogen Rh^{a}), but who have factor **Rh**$_0$, can be isosensitized to blood containing **Rh**$^{\text{A}}$. Because such an individual is **Rh**$_0$-positive, the blood cells are agglutinable by standard anti-**Rh**$_0$ serum; but the serum of this individual, even though it appears to contain anti-**Rh**$_0$, will not agglutinate the individual's own cells, which do contain **Rh**$_0$. When such a reaction occurs, investigations must be made to determine whether the patient lacks **Rh**$^{\text{A}}$, **Rh**$^{\text{B}}$, etc.

Rh-positive bloods that lack **Rh**$^{\text{A}}$ are designated as having factor **Rh**$^{\text{a}}$.

Bloods that lack **Rh**$^{\text{B}}$ have factor **Rh**$^{\text{b}}$, and so on.

Unger and Wiener[89] have found Rh_0 variants as well as standard Rh-positive bloods that lack one of the factors **Rh**$^{\text{A}}$, **Rh**$^{\text{B}}$, etc., with a higher incidence among Negroes for those lacking **Rh**$^{\text{A}}$. In most cases Rh_0 variants were associated with a variant of factor **Rh**$^{\text{A}}$ (called **Rh**$^{\alpha}$), or **Rh**$^{\text{B}}$ (called **Rh**$^{\beta}$), or **Rh**$^{\text{C}}$ (called **Rh**$^{\delta}$), and **Rh**$^{\text{D}}$ (called **Rh**$^{\gamma}$), or one or more of these factors were not demonstrable. In no instance has the **Rh**$^{\alpha}$ or **Rh**$^{\beta}$ or **Rh**$^{\delta}$ or **Rh**$^{\gamma}$ factor been found associated with a "standard" **Rh**$_0$ blood factor. Such factors react weakly or not at all with the ficinated cell technic and at times are identifiable only by the ficinated cell-antiglobulin technic.

A summary of the nomenclature regarding one of these factors follows:

Rh^{A}—all Rh agglutinogens having a "standard" **Rh**$_0$ factor and also factor **Rh**$^{\text{A}}$.

Rh^{a}—all Rh agglutinogens having a "standard" **Rh**$_0$ factor but lacking **Rh**$^{\text{A}}$.

$\mathfrak{Rh}^{\text{A}}$—all Rh agglutinogens having a variant of factor **Rh**$_0$ (generally high-grade $\mathfrak{Rh}_0$) and apparently a "standard" or possibly a high-grade variant blood factor **Rh**$^{\text{A}}$.

$\mathfrak{Rh}^{\alpha}$—all Rh agglutinogens having a variant of both blood factors **Rh**$_0$ and **Rh**$^{\text{A}}$.

$\mathfrak{Rh}^{\text{a}}$—all Rh agglutinogens having a variant of blood factor **Rh**$_0$, generally low-grade $\mathfrak{Rh}_0$, but lacking blood factor **Rh**$^{\text{A}}$.

A similar schema may be used for the other Rh factors in this category.

Unfortunately, these multiple blood factors of a single agglutinogen are antigenic, and each is capable of stimulating a corresponding specific antibody in an individual who lacks the factor. Most univalent anti-**Rh**$_0$ sera contain a mixture of Rh antibodies; therefore the rare blood cells lacking one or more of these blood factors are indistinguishable from one another unless the rare antisera specific for such blood factors are available. Similarly, anti-**Rh**$^{\text{A}}$, anti-**Rh**$^{\text{B}}$, etc. cannot be distinguished from anti-**Rh**$_0$ unless one has available, for testing, such rare cells as lack the blood factor for which the antisera are specific. In ordinary cases it is not necessary to test each Rh-positive blood to find the rare individual who lacks one or more of these blood factors, but it is important to be aware that such individuals do exist and that they could become isosensitized through transfusion or pregnancy. Lack of one or more of these blood factors occurs more frequently in Negroids than in Caucasoids and is more likely to be associated with blood containing a variant of the factor **Rh**$_0$. When Negroes receive repeated transfusions, particularly with intervals between transfusions, one must bear in mind that Caucasoid **Rh**$_0$-positive blood is almost invariably positive for all these factors, which may result in sensitization in a Negro who lacks any of the factors. In such cases either Rh-negative blood must be used or blood lacking the specific factor must be found, and there is a better chance of finding such a blood donor among Negroes than among those of other racial groups.

Factors related to rh′

(1) **rh**$^{\text{w}}$: As Wiener has pointed out, variants of each of the 3 so-called standard Rh factors have been found. In addition, each of the factors has related or associated factors. Thus **rh**$^{\text{w}}$ is one of the factors associated with **rh′**. This factor is important in that it can give rise to isosensitization in individuals who lack it.

In 1946 Callender and Race[93] detected in the blood serum of a woman with disseminated lupus erythematosus an antibody apparently formed by immunization from numerous transfusions. Her phenotype was Rh_1Rh_1 (Table 314). She had developed antibodies against factor **hr′** (a blood factor reciprocally related to **rh′**), to **N**, and to 3 previously undescribed blood factors, of which **rh**$^{\text{w}}$ was one. The superscript w was selected to designate the factor detected by her antibody because her name was Willis. It has been found that all persons who have factor **rh**$^{\text{w}}$ also possess factor **rh′**, but blood with factor **rh**$^{\text{w}}$ reacts more weakly with anti-**rh′** serum than does blood that lacks the factor. The incidence of factor **rh**$^{\text{w}}$ is about 3-7% among Caucasoids, so that it is fairly easy to find donors who lack **rh**$^{\text{w}}$.

With anti-**rh**$^{\text{w}}$ serum along with the other Rh antisera further subdivision of the Rh types has subsequently been made: rh'^{w}, Rh^{w}_1, $\text{Rh}^{\text{w}}_1\text{Rh}_2$ (as a subdivision of Rh_z); later Wiener et al.[94] showed that agglutinogens rh^{w}_y and Rh^{w}_z also exist, but these are extremely rare.

If blood of type Rh_1 has been tested for the **hr′** factor and classified as Rh_1Rh_1 (**hr′** negative) or Rh_1rh (**hr′** positive) (Table 314), tests with anti-**rh**$^{\text{w}}$ serum will further subdivide the 2 types as in Table 321. The incidence of positive reactions is considerably higher among individuals of type Rh_1Rh_1 than among individuals of type Rh_1rh.

Table 321. *Subdivision of types* Rh_1Rh_1 and Rh_1rh

Rh type	*Reaction with anti-*$\textbf{rh}^{\text{w}}$ *serum*	*Rh subtype*
Rh_1Rh_1	+	$\text{Rh}^{\text{w}}_1\text{Rh}_1$
	−	Rh_1Rh_1 (proper)
Rh_1rh	+	$\text{Rh}^{\text{w}}_1\text{rh}$
	−	Rh_1rh (proper)

In summary, it is possible for a type Rh_1 individual to have a hemolytic transfusion reaction even if given type Rh_1 blood, if the donor's cells possess **rh**$^{\text{w}}$ and the patient's cells lack this factor. Furthermore, a mother of type Rh_1 may become sensitized to blood factor **rh**$^{\text{w}}$ if present in her fetus' blood (type Rh^{w}_1 or $\text{Rh}^{\text{w}}_1\text{Rh}_2$), and the resulting antibody may give rise to erythroblastosis fetalis.

(2) **rh**$^{\text{x}}$: This is another rare blood factor which occurs only in association with **rh′**.[95] The antibody has been designated anti-**rh**$^{\text{x}}$ and the antigen it identifies, **rh**$^{\text{x}}$. With anti-**rh**$^{\text{w}}$, this antibody shows 3 sharply defined types of the Rh_1 agglutinogens: Rh^{w}_1, Rh^{x}_1, and Rh_1 proper. The last is by far the most common type.

(3) **rh**$^{\text{G}}$: There seems to be a factor shared by both **rh′** and **Rh**$_0$ in much the same manner that factor **C** of the A-B-O blood groups is shared by agglutinogens A and B (refer to p. 1472). Some **Rh**$_0$-negative individuals, sensitized with type Rh_0 blood (which lacks the **rh′** factor), have the capacity of producing

antibodies that react with type rh′ blood (which lacks the **Rh**$_0$ factor). This event has occurred following transfusions. One such serum obtained from an immunized donor[96] reacted with almost equal titer on type Rh$_0$ and rh′ blood and could be completely absorbed with either. It has been designated anti-**rh**^{G}, and the factor it identifies is called **rh**^{G}.

Factors related to rh″

(1) **rh**^{w2}: An antibody described by Greenwalt et al.[97] was found as the cause of hemolytic disease (erythroblastosis). The antigen that it identifies is called **rh**^{w2}. The cells containing this factor are agglutinated by serum containing anti-**rh**^{w2} and by some but not all anti-**rh″** sera. Up to 1958 the antigen had been found in only one family.

(2) **rh**^{u}: An antibody, anti-**rh**^{u}, was found by Race and Sanger[98] in the bloods of an Italian family. This antibody determines the antigen **rh″**^{u}, an antigen found in the agglutinogen **rh″**.

Hr FACTORS

In 1941 Levine and Javert[99, 100] reported that they had detected in the serum of an Rh-positive mother of an erythroblastotic infant an antibody that had the property of agglutinating all Rh-negative bloods. Because of the nature of this antibody, being reciprocal to Rh, it was designated "Hr" and the corresponding agglutinin as "anti-Hr." Levine reported that his anti-Hr serum gave about 30% positive reactions and that all Rh-positive bloods not agglutinated by anti-**rh′** reacted with anti-Hr. On this basis he postulated that Hr and Rh were allelic, without further clarifying his hypothesis. He also asserted that Hr incompatibility must be considered as a possibility whenever an Rh-positive mother has an Rh-negative child and that all type Rh$_1$Rh$_2$ individuals are Hr negative.

According to the observations of Wiener and others, these 2 statements have been contradicted by subsequent findings. For instance, in 1943 Race and Taylor[101] also detected in the serum of an Rh-positive mother of an erythroblastotic infant an agglutinin that reacted with all Rh-negative bloods but differed from Levine and Javert's in that it gave 80% positive reactions. As pointed out by Wiener et al.[102] Race and Taylor's serum detected the same blood property as Levine and Javert's serum, the lower percentage of positive reactions obtained by Levine being due to the use of a weaker antiserum which missed bloods heterozygous for the Hr factor.

More detailed study showed that the Hr factor was related to **rh′** in a manner similar to the relationship between M and N, that is, individual bloods are either rh′ positive or Hr positive, or both, and no blood was rh′ negative and Hr negative.[103] Wiener therefore introduced the designation **hr′** for the factor found by Levine and associates.

In 1944 Fisher[104] noted the reciprocal relationship between factors **rh′** and **hr′** and postulated the existence of 2 additional Hr factors, **hr″** and **Hr**$_0$, related reciprocally to factors **rh″** and **Rh**$_0$, respectively. In 1945 Mourant[105] announced his discovery of an antiserum that gave reactions expected of anti-**hr″**, and Wiener and Peters[106] and others found additional antisera of this specificity. Although several investigators have announced finding antisera of specificity anti-**Hr**$_0$, no confirmation of their work has been made to date, and at the present time (1963) no anti-Hr$_0$ serum is available. Anti-**hr′** and anti-**hr″** sera are used routinely in many laboratories.

The Hr factors can be defined, therefore, as factors reciprocally related to the Rh factors. They are present in agglutinogens only when the corresponding Rh factor is absent from the gene.

The **hr′** factor occurs in 80% of the population and the **hr″** in 98%; that is, only 20% lack **hr′** and only 2% lack **hr″**. The Hr factors appear to be much weaker antigens than the **Rh**$_0$ factor, which explains the relative infrequency of transfusion reactions and cases of erythroblastosis fetalis due to the Hr factors; nevertheless, they are important and must not be ignored.

Individuals who are rh′ negative are virtually always hr′ positive, and those who are rh″ negative are virtually always hr″ positive. (Exceptions are the extremely rare bloods of type $\bar{\bar{\text{R}}}\text{h}_0$, $\bar{\bar{\text{R}}}\text{h}^{w}$, and $\bar{\text{R}}\text{h}_0$). If, however, an Rh factor is present, the corresponding Hr factor may or may not be present. By using the 3 principal Rh antisera and the 2 principal Hr antisera, 18 phenotypes in the Rh-Hr system may be identified. Consult Table 322.

Other Hr factors

(1) **hr**^{S}: In 1960 Shapiro[107] announced his discovery of a new blood factor, now designated **hr**^{S}. The discovery of this new factor

Table 322. *Identification of the 18 phenotypes of the Rh-Hr system by use of 5 standard antisera*

Antisera used					
Anti-**Rh**$_0$	*Anti*-**rh**′	*Anti*-**rh**″	*Anti*-**hr**′	*Anti*-**hr**″	*Designation of types*
−	−	−	+	+	rh
−	+	−	−	+	rh′rh′
			+	+	rh′rh
−	−	+	+	−	rh″rh″
			+	+	rh″rh
−	+	+	+	+	rh$_y$rh
			−	+	rh$_y$rh′
			+	−	rh$_y$rh″
			−	−	rh$_y$rh$_y$
+	−	−	+	+	Rh$_0$
+	+	−	−	+	Rh$_1$Rh$_1$
			+	+	Rh$_1$rh
+	−	+	+	−	Rh$_2$Rh$_2$
			+	+	Rh$_2$rh
+	+	+	+	+	Rh$_z$Rh$_0$
			−	+	Rh$_z$Rh$_1$
			+	−	Rh$_z$Rh$_2$
			−	−	Rh$_z$Rh$_z$

was made during investigation of an unusual case of hemolytic disease of the newborn and was given the superscript S from the name of the mother, Shabalala. Anti-**hr**^{S} reacts like anti-**hr**″ except that certain **hr**″-positive bloods give negative reactions with anti-**hr**^{S}. Anti-**hr**^{S} has been found in all sera tested which hitherto were regarded as of anti-**hr**″ specificity. Bloods that react with anti-**hr**″ but not with anti-**hr**^{S} are designated with a caret; e.g., $\hat{R}h_0$ for the phenotype, and $\hat{R}^o$ for the gene. Bloods lacking **hr**^{S} are fairly frequent in the South African Bantu.

Discovery of the **hr**^{S} factor suggests that many human bloods previously designated Rh$_0$ were in reality $\hat{R}h_0$.

One of the most important facts in Shapiro's investigations is that the so-called "standard" anti-**hr**″ serum usually contains 2 specific antibodies, anti-**hr**^{S} and anti-**hr**″. The amount of pure anti-**hr**″ varies in these sera, a few containing potent anti-**hr**″, some having very little anti-**hr**″, and some having no detectable anti-**hr**″ after absorption with Shabalala's cells.

Sensitization of an **hr**″-positive individual with resultant anti-**hr**″-like antibody production is analogous to the anti-**Rh**$_0$-like antibody produced by some Rh$_0$-positive individuals. These antibodies were classified by Wiener and Unger (p. 1502) as anti-**Rh**^{A}, anti-**Rh**^{B}, anti-**Rh**^{C}, and anti-**Rh**^{D}. It is possible that **hr**^{S} might be the first of a series of associated blood factors related to **hr**″ in the same way as **Rh**^{A}, etc. are related to **Rh**$_0$.

Population and family studies show absence of **hr**^{S} only in certain rare rh and Rh$_0$ agglutinogens (see pp. 1597ff for discussion of genotypes):

Type $Rh_2\hat{r}h$ includes genotypes $R^2\hat{R}^o$, $R^2\hat{\mathfrak{R}}^o$, and $R^2\hat{r}$.

$\hat{R}h_0$ includes genotypes $\hat{R}^o\hat{R}^o$, $\hat{R}^o\hat{\mathfrak{R}}^o$, and $\hat{R}^o\hat{r}$.

$\hat{\mathfrak{R}}h_0$ includes genotypes $\hat{\mathfrak{R}}^o\hat{\mathfrak{R}}^o$ and $\hat{\mathfrak{R}}^o\hat{r}$.

$\hat{r}h$ has genotype $\hat{r}\hat{r}$.

It is also possible that other agglutinogens like Rh$_1$, Rh$_1^w$ may rarely lack **hr**^{S}.

By recognizing the distinction between **hr**^{S} and **hr**″, many paradoxical situations hitherto ascribed to illegitimacy or nonmaternity will doubtless be resolved.

If an antiserum containing only anti-**hr**^{S} is used as an anti-**hr**″ reagent, an Rh$_2$rh blood lacking the **hr**^{S} factor (type $Rh_2\hat{r}h$) will be mistakenly identified as type Rh$_2$Rh$_2$. Wiener had previously (1952) cautioned against the use of anti-**hr**″ sera in tests for exclusion of parentage, citing an example in which the mother was type Rh$_0$ yet her child appeared to be type Rh$_2$Rh$_2$, which, if taken at face value, would indicate that the child was not that of the mother. With the present knowledge of **hr**^{S} antibody in most anti-**hr**″ sera, it seems reasonably certain that the anomalous results reported by Wiener were

Table 323. *Reactions of cells with Rh-Hr antisera—determination of Rh-Hr phenotype using 6 antisera*

Type	*Anti-***rh′**	*Anti-***rh″**	*Anti-***Rh$_0$**	*Anti-***hr′**	*Anti-***hr″**	*Anti-***hr**	*Phenotype*
rh	–	–	–	+	+	+	rh
rh′	+	–	–	+	+	+	rh′rh
				–	+	–	rh′rh′
rh″	–	+	–	+	+	+	rh″rh
				+	–	–	rh″rh″
rh$_y$	+	+	–	+	+	+	rh$_y$rh
				+	+	–	rh′rh″
				–	+	–	rh$_y$rh′
				+	–	–	rh$_y$rh″
				–	–	–	rh$_y$rh$_y$
Rh$_0$	–	–	+	+	+	+	Rh$_0$
Rh$_1$	+	–	+	+	+	+	Rh$_1$rh
				–	+	–	Rh$_1$Rh$_1$
Rh$_2$	–	+	+	+	+	+	Rh$_2$rh
				+	–	–	Rh$_2$Rh$_2$
Rh$_z$	+	+	+	+	+	+	Rh$_z$rh
				+	+	–	Rh$_1$Rh$_2$
				–	+	–	Rh$_z$Rh$_1$
				+	–	–	Rh$_z$Rh$_2$
				–	–	–	Rh$_z$Rh$_z$

due to the fact that the serum he used contained only anti-**hr**^{S} and no anti-**hr″**.

(2) **hr:** An antiserum found by Rosenfield et al.[108, 109] in 1953 was thought at first to be the expected anti-Hr$_0$, but further studies showed that the antibody reacted with blood cells from all individuals carrying the genes R^0 or r, but did not react with blood from persons lacking these genes. The antibody was originally called anti-**f**, but the Rh-Hr symbol adopted by Wiener is anti-**hr**. The **hr** factor merely defines an additional serologic attribute which is shared by the 2 agglutinogens Rh$_0$ and rh, determined by genes R^0 and r, respectively. Anti-**hr** helps distinguish between individuals of genotypes $r'r''$ and r^yr by serologic means alone. Individuals of genotype $r'r''$ are **hr** negative and those of r^yr are **hr** positive. In individuals of type Rh$_z$Rh$_0$, the antiserum aids in distinguishing between those who do and those who do not carry gene R^z, which is of value in cases of disputed parentage. By combining the use of anti-**hr** serum with the 5 standard Rh-Hr antisera, the number of phenotypes that can be determined is extended to 20 as in Table 323.

OTHER RARE Rh-Hr BLOOD FACTORS

(1) **rh$_i$:** An additional factor has been discovered, which, in Caucasoids, is limited to the agglutinogens Rh$_1$ and rh′, determined by the genes R^1 and r', respectively. Anti-**rh$_i$** simulates anti-**rh′** in specificity because of the low incidence of agglutinogens rh$_y$ and Rh$_z$ with which only anti-**rh′** reacts.

Anti-**hr** and anti-**rh$_i$** are useful in resolving parentage disputes involving type Rh$_z$Rh$_0$ putative parents or children. For example, a type Rh$_z$Rh$_0$ person with a type rh or type Rh$_0$ child (or vice versa) would be excluded if he belonged to the common genotype R^1R^2 (or R^1r'' or R^2r') but not if he belonged to one of the rare genotypes R^zr or R^zR^0 or R^0r^y. Therefore, if the blood of such a person is rh$_i$ positive, he is excluded, whereas if it is rh$_i$ negative, he is not.

From the points of view of serology, genetics, and nomenclature, blood factors **hr** and **rh$_i$** may be considered as additional associated blood factors of previously identified Rh-Hr agglutinogens, as in the case of factors **Rh^A**, **Rh^B**, **Rh^C**, **Rh^D**, etc. At the present stage of knowledge, agglutinogen rh is known to have a total of at least 4 blood factors, **hr′**, **hr″**, **hr**, and **Hr**; whereas agglutinogen Rh$^w_1$ has a total of at least 10 blood factors: **Rh$_0$**, **Rh^A**, **Rh^B**, **Rh^C**, **Rh^D**, **rh′**, **rh$_i$**, **rh^w**, **hr″**, and **Hr**. Anti-**Hr** is presently defined as the antibody produced by sensitized persons homozygous for the so-called double bar genes, that is, persons of types $\bar{\bar{\text{R}}}$h$_0$, $\bar{\bar{\text{R}}}$h^w, etc. Double bar Rh agglutinogens (e.g., type $\bar{\bar{\text{R}}}$h$_0$) are defined as those characterized by absence of the 2 pairs of contrasting blood factors **rh′-hr′**

and **rh″-hr″**. Single bar Rh agglutinogens (e.g., type $\bar{\text{Rh}}_o$) are defined as those that lack only 1 pair of contrasting factors, namely, **rh″-hr″**. Recent work[110, 111] has shown that in chimpanzees the Rh-Hr agglutinogen resembles the single bar human Rh agglutinogen.

(2) **Rare Rh factor V, or hr^V:** In 1954 DeNatale et al.,[112] in studying blood with respect to the Rh-Hr system, showed the existence of a new factor, designated **V**. These authors concluded that the antigen is common in Negroids and rare in Caucasoids. The antigen is inherited as a mendelian dominant and has been found to belong to the Rh-Hr system. When it occurs, it is associated with genes *r* and R^o. It is common in Negroes. Geiger[113] reported a case of a white man who had had 25 transfusions without reported reactions. A sensitivity test of his serum showed the presence of antibodies reacting with factor **V**, with a titer of 16 units. The findings were verified by A. S. Wiener, who found an antibody titer of 12 units by the antiglobulin method and 8 units by the ficinated cell technic.

This antibody was found in the routine sensitivity test using the patient's serum against a cell panel suspension, in this case Hemantigen. When antibodies were found, further tests were made, using this patient's serum against specific antigen cells which are now available commercially.* This patient no doubt had received blood from a V-positive donor, which resulted in his sensitization.

ISOIMMUNIZATION (ISOSENSITIZATION) AND TRANSFUSION REACTIONS

Isoimmunization was first noted by Ehrlich and Morgenroth[114] in 1900. Whereas **heteroimmunization** refers to the production of antibodies of a member of a species against an antigen of a member of a different species, **isoimmunization** or preferably **isosensitization** refers to immunization of a member of a species by an antigen derived from another member of the same species. In other words, production of hemolysin in a rabbit after injection with sheep cells is an example of heteroimmunization.

Isoimmunization was first demonstrated when isohemolysins were produced in goats by injecting them with erythrocytes of other goats. In the Ehrlich and Morgenroth goat blood injections, the isoimmune, not the normal, isoantibodies hemolyzed the cells from the goats that had furnished the original inocula, as well as the cells of some but not of all other goats. The serum of the injected goats did not contain an autolysin, that is, it did not affect or combine with the cells of the animal in which the antibody was formed (the injected goat). The observations of Ehrlich and Morgenroth showed the possibility that a considerable number of different antigens might exist in similar cells of different individuals in a single species and also showed that an animal will not ordinarily produce antibodies to antigens that are present in its own blood. Animals can form antibodies against some of their own proteins or other components of their tissues, for example, the substance of the crystalline lens, casein,[115] and an alcoholic extract of brain,[116] but these materials are blood-foreign and thus satisfy one of the primary prerequisites for antigenicity. Immune isoantibodies have been produced in other animals and in fowls.[117]

It is difficult to perform isoimmunization experimentally in man. Thomsen[118] attempted to immunize men by intramuscular injections of incompatible blood, but no significant rise in isoagglutinative titer was observed. This apparent evidence against the isoantigenicity of the A and B group substances is, however, outweighed by the positive evidences which will be presented in reference to the Rh factor. Thomsen's negative result is probably attributable to the relatively small quantity of blood injected, or, possibly, the route of injection was influential in his negative results. Thalhimer[119] reported a case which is an example of the isoantigenicity of group substance B. Here, a boy received 300 ml. of his father's blood; cross matching showed no incompatibility, but no tests were made to determine the 2 blood groups. Only a mild febrile reaction ensued. Eighteen days later, without further tests, the father again served as donor; an alarming reaction stopped the transfusion after 150 ml. of blood had been introduced. Subsequently the bloods were grouped; the son was found to be group O and the father group B. Although no agglutinative titers were determined, it is probable that the anti-**B** normally present in group O plasma (with anti-**A**) was too weak to be detected by the ordinary test and that the primary transfusion of group B blood served as an immunizing stimulus leading probably to a marked rise in the titer of anti-**B**, which thus intensified the incompatibility. There is further evidence that **A** and **B** are isoantigenic in Jonsson's finding[120] that the maternal isohemagglutinins anti-**A** and anti-**B** are of higher titer when the corresponding antigen, **A** or **B** (not present in the mother's blood), is present in the blood of the fetus than when it is absent (transplacental isoimmunization). (Refer to blood factor **C** of the A-B-O system, p. 1472.)

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*Certified Blood Donor Service, Jamaica, L. I., N. Y., Knickerbocker in New York, and others.

Wiener[121] recorded 2 cases which illustrate isoimmunization of A_2 individuals to A_1 blood. In one the antigenic stimulus was provided by transfusion of A_1 blood; in the other the source of the antigen was a fetus in utero. Because of the presence of immune anti-$\mathbf{A_1}$ isoagglutinins in these patients' sera, only A_2 donors were used in subsequent transfusions, with satisfactory results. These workers presented evidence which indicated that had A_1 donors been used, hemolytic reactions most likely would have resulted. However, the absence of reports in recent literature indicates that subgroups A_1 and A_2, although important, are not quite as important in transfusion as formerly believed.

Further, Wiener and associates state that in selecting donors for subgroups of A and AB it is probably safe to disregard the subgroups except in the rare cases in which the patient's serum contains an irregular isoagglutinin anti-$\mathbf{A_1}$ or anti-$\mathbf{A_2}$. When the irregular isoagglutinin is present, only donors of the homologous group should be used. In this connection Gradwohl cited a case* of a recipient of the A_2B class who had a severe reaction following transfuion of A_1B blood. Later transfusion with A_2B blood resulted in no reaction. This probably corresponds to the irregular isoagglutination mentioned by Wiener.

Davidsohn[122] maintained that the subgroups A_1, A_2, A_1B, and A_2B are significant in causing some posttransfusion reactions, but direct support of this possible mechanism was lacking until Wiener's[121] report was published. Wiener analyzed a case of isoimmunization of an A_2 recipient by transfusion of A_1 blood. The anti-$\mathbf{A_1}$ behaved like a "cold" agglutinin. In another case anti-$\mathbf{A_1}$ antibody was considered to have developed in an A_2 woman as a result of isoimmunization by an A_1 fetus in utero. The general explanation was first presented by Levine and Stetson[123] as the mechanism of reactions following primary intragroup transfusions given intrapartum or postpartum.

The formation of anti-**O** isoagglutinin in low titer has been observed in a group A_1B patient.[124] This suggests that the production of immune anti-**O** isoantibodies may possibly be a factor to be considered in repeated transfusions of blood from universal donors.

Isosensitization may result not only from repeated blood transfusions but also from intramuscular injections of blood or repeated pregnancies or a combination of these, and such isosensitization may in turn be responsible for posttransfusion hemolytic reactions. As Wiener has shown, the development of isoantibodies generally requires at least 2 exposures to the antigen, with an interval of several months between each exposure. The first exposure primes the individual, and subsequent exposures stimulate the production of antibodies. In some cases a long interval is not always necessary, isosensitization resulting from continuous stimulation over a short period of time, with huge quantities of the antigen being given. Unger[125] cited the case of an Rh-negative male patient who received 5500 ml. of Rh-negative blood, and, then, because the patient was moribund and no more Rh-negative blood was available, he was given 54,500 ml. of Rh-positive blood within a period of 7 days. The transfusions were virtually continuous. Rh sensitization tests were negative until the 108th pint of blood was to have been administered, when it was found that the patient had developed univalent $\mathbf{Rh_0}$ antibodies. This is the first reported case of sensitization due to a massive dose of Rh-positive blood transfused into an Rh-negative patient. Ordinarily, an interval of a few months between exposures is necessary, for example, between 2 pregnancies or 2 transfusions bringing on sensitization.

IMPORTANCE OF Rh FACTORS

Rh factors in transfusions and retransfusions

Rh-negative individuals may become sensitized to Rh antigen in 1 of 2 ways: (1) sensitization may result from a transfusion of Rh-positive blood or (2) in women, sensitization may result from pregnancy with an Rh-positive fetus. Sensitization by either of these methods may result in a serious hemolytic reaction if transfusion with Rh-positive blood is given. Therefore, all Rh-negative women who are to be transfused **must** be given only Rh-negative blood.

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*Record of the Christian Hospital, St. Louis, Mo.

It had been observed for many years prior to 1940 that incompatible transfusion reactions occurred in a number of cases when the same donor's blood was used to transfuse a patient the second time, even though the first transfusion with such blood gave completely compatible results. These reactions took place even when both the donor and recipient were of the same blood group. After the discovery of the Rh factor by Landsteiner and Wiener, it was demonstrated that these incompatible intragroup reactions which had occurred in repeated transfusions of a patient with the same donor's blood (or blood of a compatible group) were due in most cases to incompatibility of the Rh types. Wiener demonstrated that the Rh factors are antigenic to man and that Rh-negative individuals may become sensitized to the Rh factor as a result of transfusion with Rh-positive blood.

If a patient who lacks the Rh factor is transfused with blood containing the factor, the transfused patient forms anti-Rh agglutinins which persist in his blood, and he thus has become isosensitized to the Rh factor. If he is again transfused with blood containing the Rh factor, the antibodies formed as a result of the first transfusion combine with the incoming Rh-positive donor cells and a transfusion reaction results.

The most antigenic of all the Rh factors is $\mathbf{Rh_o}$, and this factor is found in 85% of all Caucasoids. It is dangerous to give any blood containing $\mathbf{Rh_o}$ to any individual who lacks it because that person becomes sensitized to Rh. The other Rh factors are also antigenic; therefore, Rh-negative individuals must be transfused only with triple Rh-negative blood.

It is also important not to transfuse blood of Rh-sensitized donors into patients, especially into Rh-negative recipients, since introduction of antibodies that later could prove harmful is not good practice. It has been shown that antibody titer is easily enhanced with the introduction of incompatible cells into an individual with low-titer antibodies. This is the so-called **anamnestic reaction.**

In routine procedure the patient is tested for the presence or absence of $\mathbf{Rh_o}$. If this factor is not present in the patient's blood, only Rh-negative donor blood is given. A patient may lack $\mathbf{Rh_o}$ but his erythrocytes may have any of the other Rh factors. Unless a donor of the exact Rh type can be found in such cases, it is safest to transfuse this patient with triple Rh-negative (type rh) blood, that is, blood that lacks the 3 principal Rh factors, $\mathbf{Rh_o}$, **rh′**, and **rh″**.

If a patient of type rh′ is transfused with Rh_o-positive blood, this patient will become isosensitized to the $\mathbf{Rh_o}$ factor, or, in other words, his blood will contain anti-$\mathbf{Rh_o}$, and a second transfusion with Rh_o-positive blood will result in a transfusion reaction. Since it is easier to obtain type rh (triple Rh-negative) blood than either type rh′ or rh″, this patient should be given type rh blood.

If rh′ blood is given in a transfusion to an Rh-negative patient or to a patient of type Rh_2, the patient will form anti-**rh′** antibodies and therefore become isosensitized. It is for such reasons that complete Rh typing tests should be made of all donors' bloods before using them in transfusions.

Rh factors in primary transfusions of pregnant women

For the sake of simplicity of explanation in this discussion, individuals will first be considered as either Rh positive or Rh negative.

In respect to transfusing females from birth to the climacteric, if they are Rh negative and are transfused with Rh-positive blood, they may develop Rh antibodies. These antibodies may persist for life, so that if another transfusion is required and Rh-positive blood is used, severe transfusion reactions may occur as a result of the antibody-antigen reaction. Particularly the fact must be borne in mind that these Rh-negative females, who have been transfused at some time in their early life with Rh-positive blood, are isoimmunized to the Rh factor, so that when undertaking marriage and upon becoming pregnant, if the fetus is Rh positive they will most certainly have intragroup transfusion reactions if transfused with Rh-positive blood. Moreover, their chances of bearing normal infants are endangered if the fetus is Rh positive.

(The rules for the Rh factor as applied to the operation of a blood bank have been described by Unger.[126])

The Rh types of blood are hereditarily determined,[127] the $\mathbf{Rh_o}$ factor being transmitted as a simple mendelian dominant by a pair of allelic genes, *Rh* and *rh* (*Rh* determines Rh positive; *rh* determines Rh negative). Since every individual possesses a pair of genes from every series of allelic genes, one being derived from the mother and the other from the father, there are 3 genotypes possible. Rh-negative individuals belong to genotype *rhrh* and are always homozygous; Rh-positive individuals may be either homozygous (genotype *RhRh*) or heterozygous (genotype *Rhrh*). Therefore, 2 Rh-negative parents can have only Rh-negative children. If one parent is Rh negative and the other is Rh positive, the children will all be Rh positive if the Rh-positive parent is homozygous. If the Rh-positive parent is heterozygous, there is equal chance that the children may be Rh positive or Rh negative; or as it is usually stated, one-half of the children will be Rh positive and one-half will be Rh negative. When both parents are Rh positive, all the children will be Rh positive, except when the parents are both heterozygous, in which case one-fourth of the children will be Rh negative.[128] For a fuller discussion see heredity of the blood groups, pp. 1597ff and 1606-1612.

In 1940 Levine et al.[129, 130] noted that sera of patients who had had repeated miscarriages or abortions contained atypical isoagglutinins related to the Rh factor. In 1941 Katzin found that the serum of the patient previously described by Levine and Stetson[123] agglutinated the same cells as the newly discovered anti-rhesus rabbit serum. Levine et al.[131] called attention to the fact that a diagnosis of erythroblastosis fetalis had been made on some of the dead fetuses which came from the union of Rh-positive and Rh-negative persons. Vogel[132, 133] noted that the majority of the mothers had Rh-negative blood. Burnham[134] and Levine[135, 136] further called attention to the relationship of the Rh factor to erythroblastosis. The doctrine that Rh-positive fetal cells entering the maternal blood stimulate the formation of anti-Rh agglutinins is now accepted. Such agglutinins pass back to the fetus and then clump and destroy the fetal erythrocytes.

An excellent description of what takes place in the placenta itself was given by Pieri and Schwartz[137] (Figs. 488 and 489). The medium for the interchange of gases, water, and nourishment between mother and baby is the fragile membrane of the placental villus, a trophoblastic projection of the chorion frondosum itself. Through an arteriole, each villus is supplied with fetal blood by a branch from one of the umbilical arteries, while a corresponding venule eventually returns the blood to the fetal circulation. The delicate placental villi are bathed in pools of maternal blood in the intervillus spaces. The maternal blood is carried to these spaces by minute branches of the uterine artery, which are usually found in relation to the decidual septa, while corresponding veins return it eventually to branches of the uterine vein. Constant circulation is assisted by the uterine contractions which occur at intervals throughout every pregnancy. Normally, the blood of the child circulates inside the villus, while the maternal blood circulates outside this delicate structure. The relationship is so close that osmosis readily takes place through the villus wall. It has been estimated that if all the villi of a placenta at full term were placed end to end, their combined length would exceed 11 miles.

That the placenta is extremely susceptible to the effects of tumors, trauma, and disease has long been known. This is not a surprising observation in view of the complex structure and function of this organ and the fact that the more complex an organ the more delicate and vulnerable to injury it becomes.[138] Careful scrutiny of the placenta[139] will reveal in nearly every instance the unmistakable evidences of one or many areas of old or recent pathologic alteration. Such scars, varying in size from pinhead to several c.mm., have been designated by a confusion of terms, such as infarction, hepatization, placentitis, necrosis, apoplexy, etc. Actually each such scar represents merely the evidence of a previous placental injury. Since the most delicate portion of the placenta is the villus itself, this structure is the most susceptible to injury as a result of apoplexy, hemorrhagic infarction, or direct trauma. Whatever the causative factor, any violent break in the continuity of the vessels of one or more villi will result in hemorrhage of fetal blood cells into the maternal circulation. From here the fetal blood elements are carried into the uterine veins and thence into the general circulation of the mother. Such a hemorrhage may be minute and apparently insignificant, or it may be of considerable size.[140] (In fact, Wiener has described an anemia of newborn babies due to occult placental bleeding of fetal blood into the maternal circulation.[141]) Either may produce most serious sequelae.

Now, if the fetus' cells contain the Rh antigen (Rh positive) while those of the mother are without the antigen (Rh negative), a variable degree of isoimmunization may result. The Rh-positive cells of the fetus stimulate within the Rh-negative mother formation of the specific Rh antibody (anti-Rh agglutinin).

If the mother now needs a transfusion and Rh-positive donor blood is used, there follow

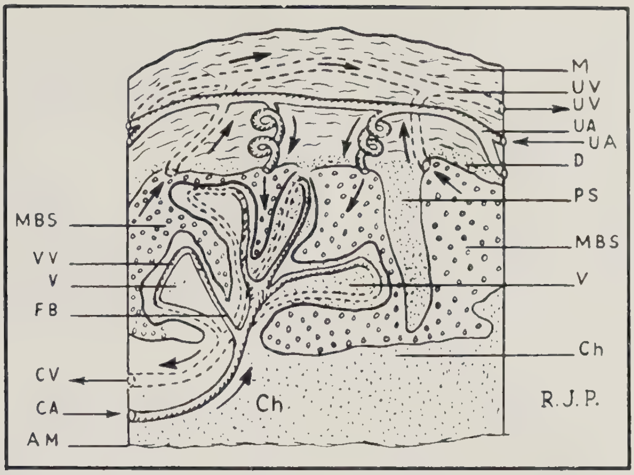

Fig. 488. Diagrammatic section through a human placenta. **M**, Uterine muscle; **UV**, branch of uterine vein; **UA**, branch of uterine artery; **D**, decidua; **PS**, decidual septum; **MBS**, maternal blood sinus; **V**, villus; **Ch**, chorion frondosum; **AM**, amnion; **CA**, branch of umbilical artery; **CV**, branch of umbilical vein; **FB**, villus branch of umbilical artery; **VV**, vein withdrawing blood from villus. (From Pieri, R. J., and Schwartz, R. C.: New York J. Med. **46:** 387, 1946.)

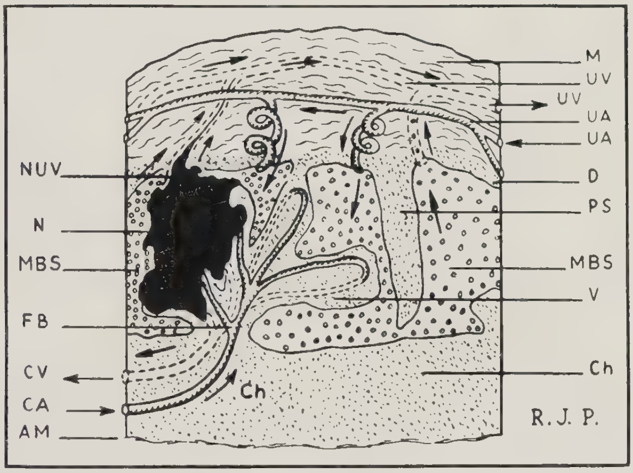

Fig. 489. Diagrammatic illustration of rupture of placental villus. **N**, Fetal blood from villus vessels pouring into maternal blood sinus, **MBS.** Fetal blood enters maternal uterine vein at **NUV**, to gain access to general circulation of the mother (see text). (From Pieri, R. J., and Schwartz, R. C.: New York J. Med. **46:**387, 1946.)

hemolysis and agglutination of the donor's cells, with resulting renal damage or even death, from the action of the mother's isoantibodies (anti-Rh) on the incoming Rh-positive cells. Therefore, in all primary transfusions of Rh-negative pregnant or postpartum women use Rh-negative donors.

Rh factors in erythroblastosis fetalis*

The Rh antibody formed in the blood serum of the Rh-negative mother by virtue of the presence of an Rh-positive fetus (see previous discussion on Rh factors in primary transfusions of pregnant women) is readily transmissible (when it is univalent) as are many toxins and antitoxins, through the placental filter (the walls of the villi) into the circulation of the infant in utero. It is the action of this antibody upon the Rh-positive cells of the baby that produces a disease entity recognized as isohemolytic disease or erythroblastosis fetalis which in about 90% of the cases is due to incompatibility of the Rh factors. In most of the remaining 10% the disease apparently depends upon similar genetically re-

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*Refer to Wiener, A. S.: Advances in blood grouping, New York, 1961, Grune & Stratton, Inc.

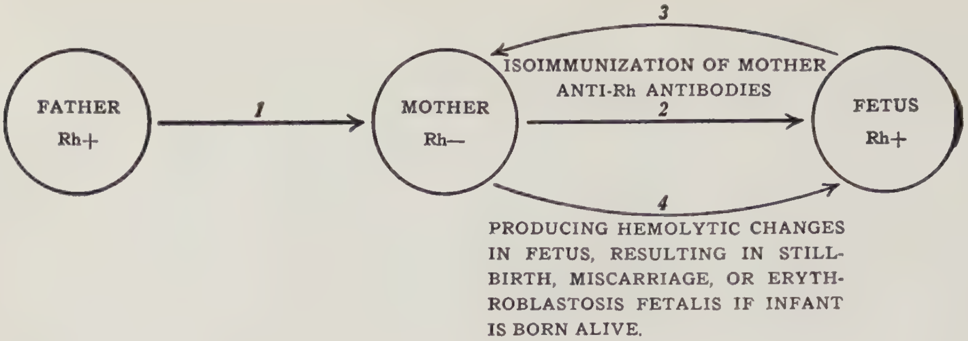
FATHER
Rh+
1
MOTHER
Rh—
3
ISOIMMUNIZATION OF MOTHER
ANTI-Rh ANTIBODIES
2
FETUS
Rh+
4
PRODUCING HEMOLYTIC CHANGES IN FETUS, RESULTING IN STILLBIRTH, MISCARRIAGE, OR ERYTHROBLASTOSIS FETALIS IF INFANT IS BORN ALIVE.

Fig. 490. Diagram of Rh phenomenon in pregnancy.

lated antigens (such as those designated as Hr or other blood group antigens) present in the baby's cells but absent in those of the mother.

Fig. 490 illustrates what takes place in pregnancy when an Rh-negative woman is mated to an Rh-positive man. The Rh positive fetus stimulates formation of Rh antibodies in the mother (isoimmunization). Anti-Rh antibodies from the mother pass through the placenta, producing hemolytic changes in the fetus. This may result in stillbirth or in erythroblastosis fetalis if the infant is born alive. It must be noted, as Wiener first pointed out, that only the univalent antibodies enter the fetal circulation so that if bivalent antibodies alone are formed by the mother, the infant is not affected.

Although the mating of Rh-negative women with Rh-positive men occurs in 1 out of 10 marriages, congenital hemolytic disease affects only 1 in about 250 newborn infants. This suggests that only about 1 in 25 Rh-negative women becomes sensitized when bearing an Rh-positive fetus, just as in sensitization by blood transfusion. The first-born child is rarely affected, because it takes at least one pregnancy and sometimes more before a sufficient degree of sensitization develops. In most of the rare cases in which the first-born infant was affected, a careful history has revealed that the woman had previously received a transfusion of Rh-positive blood. According to Wiener, this serves to emphasize the importance of performing Rh tests before giving blood transfusions to women in the childbearing age, as well as to young girls, because a transfusion of the wrong Rh type blood could ruin a woman's chances of having a normal child. (Refer to pp. 1488 and 1489 and below for a discussion of univalent antibodies.)

If the infant is transfused with Rh-positive cells, the Rh antibodies derived from the mother, which are in excess of those that coat the infant's cells, react with the incoming Rh-positive donor blood cells and a reaction ensues.

Therefore, in all pregnancies in which the mother is Rh negative and the father is Rh positive be prepared for an erythroblastotic infant. If a direct antiglobulin test (Coombs test) of the infant's cells is positive at birth, transfuse the infant immediately upon birth with Rh-negative donor blood, or, preferably, perform an exchange transfusion using Rh-negative blood. If no Rh-negative donor is available, use the washed red cells of the Rh-negative mother. Washing the cells removes the Rh antibodies. In emergencies, however, the mother's whole blood may be used. See pp. 1537ff for exsanguination or exchange transfusions.

NATURE OF ANTIBODIES*

Antibodies appear in the blood in 2 principal forms: (1) **univalent**—also called conglutinating, blocking, incomplete, or coating antibodies—and (2) **bivalent**—also called agglutinating, complete, precipitating antibody. The univalent antibodies occur later in the course of immunization than do the bivalent antibodies, are relatively thermostable, and readily pass through the intact placenta. Many of the univalent antibodies combine with and coat their specific cells when such cells are suspended in a saline medium, but the cells do not clump. When high molecular colloids or proteins are present, the univalent antibodies cause the cells to clump. On the other hand, bivalent antibodies will react with cells that are suspended in saline media as well as with cells suspended in high molecular colloids. Bivalent antibodies are usually produced early in the course of immunization, are relatively thermolabile, and are effectively held back by an intact placenta.

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*See Table 311.

The use of the terms univalent and bivalent is not intended to imply chemical valence. These terms were coined by A. S. Wiener in order to impart a visual concept of in vitro reactions. See Figs. 484-487 and 494.

It must be assumed that the bivalent molecule, which has a probable molecular weight of 930,000 against a 155,000 molecular weight of the univalent molecule, is larger and therefore cannot transverse the placental barrier. In syphilis the bivalent antibody is a flocculating antibody, whereas the univalent antibody is a complement fixing antibody. The antisheep hemolysin used in complement fixation tests is regarded as a univalent antibody since it requires complement to complete its reaction with sheep cells.

The anti-**A** and anti-**B** agglutinins are in most cases bivalent antibodies, whereas anti-**C** (of the A-B-O system) is more often univalent. Rh antibodies may be univalent or bivalent, or both may be present in the same serum. For this reason tests for both types of antibodies should always be made with unknown serum when sensitivity testing is done.

Univalent antibodies "coat" cells but lack the capacity to clump them in saline medium, and since the antibodies are gamma globulins, this coating can be detected by the antiglobulin method.

Wiener[143] described the difference between the 2 varieties of antibodies and their reaction in vitro as follows:

Anti-Rh agglutinins (bivalent) + Rh-positive cells = Rh agglutination.

Anti-Rh glutinins (univalent or blocking antibodies) + Rh-positive cells + X protein = Rh conglutination.

The third component listed here, X protein, is a normal component of blood serum. It is adsorbed onto the specifically sensitized red cells, causing them to stick together. This X protein is distinct from complement. It resists heating at 60° C. and is a colloidal constituent or conglutinin in the plasma, a large molecular complex of albumin, globulin, fibrinogen, and phospholipid. X protein, although heat-resistant, dissociates readily into its constituent smaller molecules of albumin and globulin upon dilution of the plasma or serum with water. Use of saline to suspend cells or to dilute serum may either partially or completely dissociate the X protein. Thus, in order to carry out conglutination tests, any crystalline solution must be strictly avoided.

Chapter 75

Tests for the determination of the Rh-Hr factors

ADDINE G. ERSKINE

When determining blood groups and types, there must be available a battery of antisera, that is, sera containing specific antibodies. Each antiserum contains only antibodies specific for the blood factor in question and no other antibodies. The blood factors detected by the various antisera are not all independent of one another but fall naturally into sets which are known as blood group systems.

DETECTION OF Rh FACTORS IN BLOOD CELLS

A requisite for accurate and successful Rh testing is an efficient anti-Rh serum. Originally the source of such serum was from women who had given birth to erythroblastotic babies or who had had spontaneous abortions or stillbirths resulting from Rh incompatibility. More recently, a number of reliable biological houses* have produced excellent anti-Rh testing sera by immunizing normal male professional blood donors (p. 1532). The original anti-Rh serum was produced in animals by inoculating them with rhesus monkey blood, but such sera have some disadvantages—the amount of blood obtainable is small, absorption with Rh-negative cells is necessary to remove undesirable antibodies, and anti-Rh sera from animals immunized with rhesus blood contain only the antibody corresponding to anti-**Rh**$_0$.

Two types of anti-Rh sera are available. One, called conglutinating serum, may be used for blood cells suspended in their own serum or plasma or fortified with conglutinin. It is not reactive with saline-suspended cells. Such sera are used in the open slide methods and for detecting **Rh**$_0$ variant, as well as for coating Rh-positive cells. The other type, called agglutinating serum, may be used for cells suspended in saline as well as in conglutinin. Such antisera are used for the tests made in tubes in which saline-suspended cells are used. Either can be used for enzyme-treated cells.

Conglutinating antisera of 3 Rh specificities* are available for Rh testing: (1) anti-**Rh**$_0$ will detect the **Rh**$_0$ factor in types Rh$_0$, Rh$_1$, Rh$_2$, and Rh$_z$; (2) anti-**Rh**$_0$′ will detect both the **Rh**$_0$ and the **rh′** factors found in types Rh$_0$, Rh$_1$, Rh$_2$, Rh$_z$, rh′, and rh$_y$; and (3) anti-**Rh**$_0$″ will detect both the **Rh**$_0$ and the **rh″** factors found in types Rh$_0$, Rh$_1$, Rh$_2$, Rh$_z$, rh″, and rh$_y$. Manifestly, if the unknown blood cells have the **Rh**$_0$ factor, all 3 antisera will cause their agglutination. But if the **Rh**$_0$ factor is not present in the cells while either **rh′** or **rh″** is present, or if both **rh′** and **rh″** are present, then one or the other of antisera (2) or (3) above will cause their agglutination. These so-called "slide" sera are used primarily for screening tests to determine whether or not **Rh**$_0$ is present, or, if it is absent, whether or not **rh′** or **rh″** is present. Actual identification of the 8 primary Rh blood types cannot be made with these conglutinating sera. Tube tests must be made, using agglutinating sera of different specificities, and each factor determined separately.

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*Certified Blood Donor Service, Inc., Jamaica, L. I., N. Y,; High Titer Serum Laboratories, New York, N. Y.; Ortho Pharmaceutical Co., Raritan, N. J.; Knickerbocker Laboratories, New York, N. Y.; Hyland Laboratories, Los Angeles, Calif.

*This does not include conglutinating sera of anti-**hr′** and other specificities.

Anti-$\mathbf{Rh}_0$ serum shows 85% of all bloods from Caucasians tested to be Rh positive; anti-$\mathbf{Rh}_0'$, when used alone shows slightly less than 87% of all bloods from Caucasians to be Rh positive; whereas anti-Rh_0'', if used alone, will show about 85.5% of all Caucasian bloods to be Rh positive.

Agglutinating sera for Rh typing are of 3 main specificities: anti-$\mathbf{Rh}_0$, which detects the $\mathbf{Rh}_0$ factor (present in 85% of Caucasoids), anti-$\mathbf{rh}'$, which detects the $\mathbf{rh}'$ factor (present in 70% of Caucasoids), and anti-$\mathbf{rh}''$, which detects the $\mathbf{rh}''$ factor (present in 30% of Caucasoids). (In addition to tests with these 3 antisera, a test is also made for the $\mathbf{Rh}_0$ variant, using anti-$\mathbf{Rh}_0$ conglutinating serum plus an antiglobulin test.) If blood is type Rh_1 (Rh_0'), the cells are agglutinated in the tube test by both the anti-$\mathbf{Rh}_0$ and the anti-$\mathbf{rh}'$ sera; if it is Rh_2 (Rh_0''), it is clumped by both anti-$\mathbf{Rh}_0$ and anti-$\mathbf{rh}''$; and if it is Rh_z (Rh_0'''), it is clumped by all 3 of these antisera.

Slide method

When testing the patient, use the anti-$\mathbf{Rh}_0$ serum as a routine procedure. If this recipient is Rh negative by the slide test, give only Rh-negative donor blood in a transfusion. If the blood proves to be Rh-positive, give Rh-positive blood.

When testing donors, all 3 conglutinating antisera should be used routinely as previously explained. If the donor's blood is aglutinated by the anti-$\mathbf{Rh}_0$ serum, all 3 tests will be positive. If the test with anti-$\mathbf{Rh}_0$ is negative but either or both the other 2 tests are positive, this donor is considered Rh positive and the blood is **not** to be used for Rh-negative recipients. If positive by any of the 3 tests, the blood is given only to Rh-positive recipients.

If the results with all 3 antisera are negative, the blood may be used for Rh-negative recipients, provided that tube antiglobulin tests prove the absence of $\mathfrak{R}\mathbf{h}_0$.

Equipment:

1. A special viewing box is used for this test. This box is fitted with an electric light globe which will bring the temperature on the plate to 45°-47° C. See Figs. 476 and 477. Since some time is required to bring the temperature on the glass plate to the proper degree, the light should be on at all times.
2. Ordinary glass microscope slides are used.
3. In addition to the antisera, whole blood taken in a special dry anticoagulant is used (pp. 1461 and 1462). If clotted blood is submitted, break up the clot and suspend the cells to a concentration of 50% in their own serum. Do not use blood taken in a crystalline solution (saline, sodium citrate, liquid oxalate, etc.). The patient's blood should be centrifuged and enough plasma removed to bring the cell concentration to 50%.

Reagents: For recipients, use anti-$\mathbf{Rh}_0$ slide (conglutinating) serum alone; use all 3 antisera, anti-$\mathbf{Rh}_0$, anti-$\mathbf{Rh}_0'$ and anti-$\mathbf{Rh}_0''$ sera for donors. (If blood bank blood is used, the exact type of blood of the donor is often given on the blood bottle. This can be detected by use of the tube-agglutinating method. See below.)

Procedure: Carry out the method for each antiserum used.

1. Make a large oval ring on a glass slide, using a red wax pencil. The oval is about 1½ × ¾ in.
2. Label with the name of the individual being tested and the serum used. See Fig. 482.
3. Place 2 drops of whole blood on the slide.
4. Add 1 large drop of antiserum (anti-$\mathbf{Rh}_0$ if this is the recipient).
5. Prepare similar slides for each antiserum used to test the blood.
6. Mix each with a separate wooden applicator, spreading the mixture over the entire surface of the oval ring.
7. Place on a preheated viewing box and rock back and forth, observing for clumping or lack of clumping.
8. At the end of 3-5 min. tip the box and add a drop of saline to the top of the slide so that it runs down into the mixture. Rouleaux will be broken up by the saline, but true clumping will not.
9. Read macroscopically only; do not read under a microscope.
10. Wait for 1 or 2 min. before taking the final reading.
11. Report clumping as positive, lack of clumping as negative.

Controls: At the same time run parallel tests using known Rh-positive and Rh-negative whole blood as controls. If these react correctly, the results in the tests may be reported. If the controls give incorrect reactions, do not report the results of the tests until new antisera are obtained and the tests and controls have been repeated.

Results possible: For the recipient, see Table 324; for the donor, see Table 325.

Tube method

This test should be made routinely on all Rh-negative donors and preferably also on all Rh-negative patients.

Reagents: Anti-$\mathbf{rh}'$ saline agglutinating serum, anti-$\mathbf{rh}''$ saline agglutinating serum, anti-$\mathbf{Rh}_0$ saline agglutinating serum, anti-$\mathbf{Rh}_0$ conglutinating (slide test) serum. The anti-$\mathbf{Rh}_0$ conglutinating serum is used to detect the Rh_0 variant $\mathfrak{R}\mathbf{h}_0$ or D^u).

In addition to these sera, a 5% suspension of the patient's (or donor's) cells in saline is needed. Make the suspension as directed on p. 1463, wash once in saline, and resuspend to a 5% suspension in saline.

Use tubes 7.5 cm. × 8 mm., 4 for each test. Refer to Figs. 491 and 492.

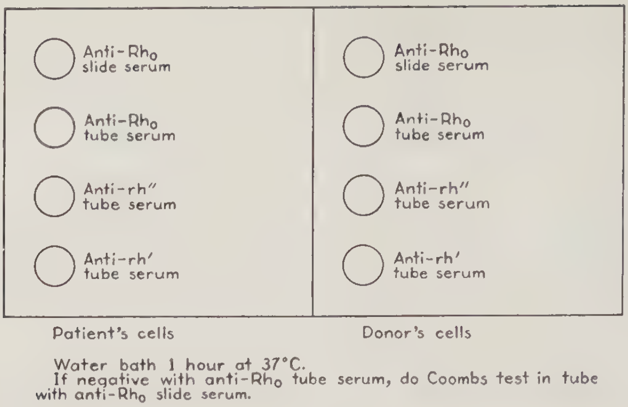
Anti-Rho slide serum
Anti-Rho tube serum
Anti-rh″ tube serum
Anti-rh′ tube serum
Anti-Rho slide serum
Anti-Rho tube serum
Anti-rh″ tube serum
Anti-rh′ tube serum
Patient's cells
Donor's cells
Water bath 1 hour at 37°C.
If negative with anti-Rho tube serum, do Coombs test in tube with anti-Rho slide serum.

Fig 491. Tube test for Rh factors.

Table 324. *The recipient*

*Whole blood plus anti-***Rh**$_0$ *serum*	*Conclusions*
+	**Rh**$_0$ positive: give Rh-positive blood
–	**Rh**$_0$ negative: give Rh-negative blood

Table 325. *The donor*

*Whole blood plus anti-***Rh**$_0$ *serum*	*Whole blood plus anti-***Rh**$_0$′ *serum*	*Whole blood plus anti-***Rh**$_0$″ *serum*	*Conclusions*
–	–	–	Type rh (Rh negative)*
+	+	+	Rh positive containing **Rh**$_0$
–	+	–	**rh**′ factor present†
–	–	+	**rh**″ factor present†
–	+	+	**rh**′ and **rh**″ factors present†
+	–	–	Error
+	–	+	Error
+	+	–	Error

*Tests should be made for the **Rh**$_0$ variant, $\mathfrak{Rh}_0$.

†Give this blood only to Rh-positive patients. This individual should receive only Rh-negative blood unless the exact type is available. Tests should be made for the $\mathfrak{Rh}_0$ factor.

Procedure:

1. Label tubes with the identification of the individual being tested and the identity of the antiserum.
2. Place 1 drop of cell suspension to be tested in each of 4 tubes in a rack. Use the same dropper held at the same angle so that the drops in each tube will be approximately the same size.
3. Add:
   - 1 drop of anti-**rh**′ saline agglutinating serum to tube 1
   - 1 drop of anti-**rh**″ saline agglutinating serum to tube 2
   - 1 drop of anti-**Rh**$_0$ saline agglutinating serum to tube 3

Fig. 492. Tube test for Rh factors in cell suspensions.

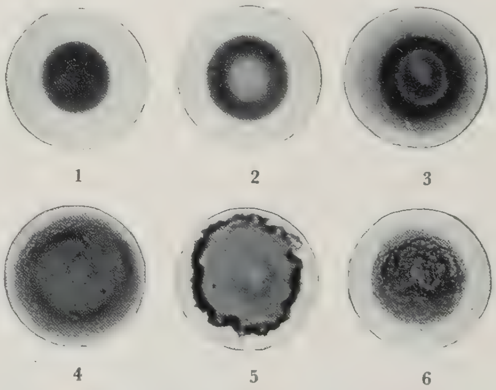

Fig. 493. Magnification 1:2. **1** and **2,** Negative reactions; the inner light disk in **2** is due to slight convexity in the bottom of the tube. **3,** Faintly positive reaction. **4,** Weak reaction. **5** and **6,** Typical positive reactions. (From Landsteiner, K., and Wiener, A. S.: J. Exper. Med. **74:**312, 1941.)

1 drop of anti-**Rh**$_0$ slide agglutinating (conglutinating) serum to tube 4.

4. Mix and incubate in a water bath at 37° C. for 1 hr.
5. Tip the rack and take a preliminary reading from the bottoms of the tubes (Fig. 493). Negative reactions come down in a pear shape, whereas positive reactions tend to remain round.
6. Read over a lighted box, spreading the mixture thin in the tube. Read under a scanning lens of a microscope, reporting clumping as positive and lack of clumping as negative. The reactions are easier to read if the tubes are centrifuged at low speed for 1 min. or if they are left at room temperature for 1 hr. before reading.

If the result in tube 3 (anti-Rh$_0$ saline agglutinating serum) is negative, use tube 4 for an antiglobulin (Coombs) test as follows:

7. Wash the cells in tube 4 three times with saline. After the last washing remove all traces of the saline. This can be accomplished by centrifuging at high speed, turning the tube upside down after the cells have been packed and striking the lip of the tube on a gauze pad while the tube is still upside down.
8. Shake thoroughly and incubate for 5 min. in a water bath at 37° C.
9. Add 1 drop of antiglobulin (Coombs) serum.
10. Mix thoroughly and centrifuge at 500-1000 r.p.m. for 2 min.
11. Read over a lighted box and under a scanning lens. Consult Table 326 for results.

If the results in tube 3 (anti-**Rh**$_0$ saline agglutinating serum) **are positive,** omit the Coombs test on tube 4 because it will always be positive under these circumstances.

If the anti-**rh'** serum clumps the cells, **rh'** factor is present.

If the anti-**rh''** serum clumps the cells, **rh''** factor is present.

If the anti-**Rh**$_0$ saline agglutinating serum clumps the cells, **Rh**$_0$ factor is present.

If the anti-**Rh**$_0$ saline agglutinating serum fails to clump the cells but the Coombs test is positive in tube 4 (slide agglutinating anti-**Rh**$_0$ serum), the **Rh**$_0$ variant is present. This is reported by using the box as given in Table 326, as suggested by Unger.

Rapid tube method

1. Place 1 large drop of conglutinating anti-**Rh**$_0$ testing serum (slide serum) in a small tube, inside diameter 7-8 mm.
2. Dip a wooden applicator into the sample of blood to be tested, either whole oxalated blood or clotted blood, to pick up some of the red cells.
3. With the applicator transfer enough unknown cells to the tube containing the antiserum to prepare a 2% suspension of red cells in the serum.
4. Place the tube in a water bath at 37° C. for a few minutes to warm it and then centrifuge at 500 r.p.m. for 1 min.
5. Shake gently to free the sediment. In positive reactions the sediment comes away in a single clump or several large clumps. In negative reactions the cells separate to form a homogeneous suspension.
6. Check the readings microscopically, using a scanning lens.
7. Instead of centrifuging the tubes, allow them to stand for 1 hr. in a 37° C. water bath. Then shake to dislodge the sediment and read the reactions macroscopically and under a scanning lens.

Table 326. *Results possible with the tube test for Rh factors**

*Cells + anti-***rh'** *serum*	*Cells + anti-***rh''** *serum*	*Cells + anti-***Rh**$_0$ *serum*	*Cells + anti-***Rh**$_0$ *slide serum (plus Coombs test)*	*Conclusions (Rh type)*
–	–	–	$\underline{-\vert}$ –	rh
+	–	–	$\underline{-\vert}$ –	rh'
–	+	–	$\underline{-\vert}$ –	rh''
+	+	–	$\underline{-\vert}$ –	rh$_y$
–	–	+		Rh$_0$
+	–	+		Rh$_1$
–	+	+		Rh$_2$
+	+	+		Rh$_z$
–	–	–	$\underline{+\vert}$ –	$\mathfrak{R}$h$_0$
+	–	–	$\underline{+\vert}$ –	$\mathfrak{R}$h$_1$
–	+	–	$\underline{+\vert}$ –	$\mathfrak{R}$h$_2$
+	+	–	$\underline{+\vert}$ –	$\mathfrak{R}$h$_z$

*After Lester J. Unger, New York City.

$\underline{-\vert}$ means that the original test was negative and
–
also the Coombs test (in the box).

$\underline{+\vert}$ means that the original test was negative and
–
the Coombs test was positive (in the box).

Positive and negative controls must be run at the same time as the unknowns.

The glassware must be clean and **dry.** Conglutinin, present in the testing serum, is sensitive to dilution with aqueous solutions and dissociates into its various serum protein components.

The blood specimens must be fresh or properly stored in a refrigerator. Avoid freezing or exposure of the blood to warm temperatures. Do not use specimens diluted with aqueous solutions.

Temperatures of the water bath may be as high as 40° C., but above this the red cells are damaged and the reactions are weak.

Avoid vigorous centrifuging.

Sources of error: Wiener has given the following sources of error in performing the saline agglutinating tests for Rh factors:

1. Poor blood samples:

(a) Whole blood keeps well for 5-7 days when refrigerated and may be taken either as a clot or mixed with dry oxalate. The blood suspensions do not keep and must be made fresh. The cell suspensions may deteriorate in warm weather within a few hours.

(b) Hemolyzed blood should not be used. Do not use wet syringes or alcohol in the syringes, and avoid too hot or too cold apparatus.

(c) Blood obtained by inadequate finger puncture may be mixed with tissue juices and gives poor results.

(d) Infected blood samples give weak reactions or false (bacteriogenic) clumping.

(e) When in doubt obtain a fresh blood sample by venipuncture.

2. Too weak a blood cell suspension gives poor results, as does too strong a suspension.

3. Use 7-8 mm. tubes, since wider tubes favor evaporation and the reactions are likely to be indistinct. Too narrow tubes are difficult to manage.

4. Maintain a constant 37° C. water bath. Lower temperatures give weaker reactions, and too high temperatures damage cells and serum.

5. Handle the tubes gently since rough handling breaks up the reaction. Roll or twist the tubes to dislodge the sediment but do not shake. When possible read the reactions in the original tube.

6. Take care not to mistake swirls of cells and small clots for agglutination. In true positive reactions the cells are strongly clumped with only a few free cells between the clumps.

7. Do not use citrate solution for preparing the blood cell suspension. Use physiologic saline.

8. Soapy, dirty, or old etched tubes must be avoided. Use only scrupulously clean tubes.

9. Use round-bottom or conical tubes only.

10. Do not use contaminated or outdated sera.

11. Always include at least one known positive specimen and one known negative specimen as controls.

Rapid mass Rh_o typing with bromelin—Pirofsky, Nelson, and Pittenger[144]

Bromelin consists of a group of proteolytic enzymes obtained from the pineapple stem. Under the trade name Bromezyme, it is commercially available* ready for use.

Standard bromelin solution:

1. Prepare a 0.5% bromelin solution by dissolving 0.5 gm. bromelin in 90 ml. physiologic saline and 10 ml. Sørensen phosphate buffer, 0.15M, pH 5.5.
2. Add 0.1% sodium azide and 0.05% actidione as preservatives.
3. Store at 4° C. Prepare fresh solutions monthly.

The commercial preparation may be used in place of this formula.

Procedure:

1. Mix 1 part anti-**Rh₀** conglutinating (slide test) serum with 5 parts saline.
2. Place 2 drops of diluted antiserum in a 10 × 75 mm. tube.
3. Add 1 drop of bromelin solution.
4. Insert a wooden applicator into the blood clot or oxalated whole blood and mix the adherent blood cells into the bromelin solution.
5. Shake for 30 sec. in a Kahn shaker.
6. Centrifuge **immediately** for 1 min. at 1000 r.p.m.
7. Examine macroscopically for agglutination or lack of agglutination, first gently dislodging the sediment. A positive reaction means that **Rh₀** is present on the unknown cells; a negative reaction means that the cells are negative for **Rh₀**.

Bromelin apparently functions by modifying the surface of the erythrocytes. Essentially identical results are obtained whether erythrocytes are pretreated with bromelin and the bromelin then washed from the test system or if bromelin is present in the test system. False positive bromelin reactions in systems that did not contain erythrocyte antibodies were seen at the following concentrations: bovine albumin 15%, human albumin 12.5%, electrophoretically pure gamma globulin 5%, polyvinylpyrrolidone 1.25%, and dextran 1.5%.

DETECTION OF Hr FACTORS IN BLOOD CELLS

If Hr tests are to be made, remember that these will always be positive on Rh-negative persons; therefore, to conserve anti-Hr sera it is best to wait for the results of the Rh tests before making Hr tests.

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*Specific Serums, Inc., Hoboken, N. J.

After a blood sample has been classified into one of the Rh blood types (p. 1517), selected Hr tests are made with the aid of anti-Hr sera. In order to avoid unnecessary Hr testing and to conserve the valuable Hr antisera, only bloods of Rh_1 and rh′ need be tested with anti-**hr′** serum, and likewise only bloods of types Rh_2 and rh″ need be tested with anti-**hr″**. (There are exceptions to this, for example, bloods containing $\overline{\mathbf{R}}\mathbf{h}_0$ and $\overline{\overline{\mathbf{R}}}\mathbf{h}_0$.) By using these antisera along with the various anti-Rh sera, the Rh-Hr subtypes are designated as follows:

Rh_1, hr′ negative is called $\mathrm{Rh}_1\mathrm{Rh}_1$.
Rh_1, hr′ positive is called $\mathrm{Rh}_1\mathrm{rh}$.
rh′, hr′ negative is called rh′rh′.
rh′, hr′ positive is called rh′rh.
Rh_2, hr″ negative is called $\mathrm{Rh}_2\mathrm{Rh}_2$.
Rh_2, hr″ positive is called $\mathrm{Rh}_2\mathrm{rh}$.
rh″, hr″ negative is called rh″rh″.
rh″, hr″ positive is called rh″rh.

The above designations are phenotypes and not genotypes. (See Table 314, p. 1495.)

Anti-Hr testing sera are fortified with bovine albumin. Despite this fortification, umbilical cord blood or blood from newborn infants not infrequently give weak reactions because of their low concentration of conglutinin. In such cases, if in doubt, centrifuge the blood sample, remove the plasma, and replace it with an equal volume of compatible normal adult plasma; then resuspend the cells and proceed with the test. This method will also help eliminate doubtful reactions due to pseudoagglutination occurring with blood from patients who have certain hyperproteinemic states, such as myeloma, or in diseases associated with a high fibrinogen content of the blood, such as pneumonia and pregnancy.

Retest all negative and weak reacting bloods by the tube method, using anti-Hr agglutinating sera when available.

Open slide method for Hr factors

1. Prepare oxalated whole blood as directed on p. 1464.
2. Obtain slide sera (conglutinating sera), anti-**hr′** and anti-**hr″**.
3. Prepare 2 slides by marking them with a large oval, using a red wax pencil. Label properly.
4. Place 2 drops of oxalated whole blood on each slide.
5. Add 1 drop of anti-**hr′** serum to one slide and 1 drop of anti-**hr″** serum to the other.
6. Mix each with a separate applicator and spread over the area of the oval.
7. Place on a preheated lighted viewing box and rock back and forth for 2 or 3 min. Do not allow to dry.
8. Add a drop of saline after the reaction has had time to take place and let it run down the specimen. If rouleaux are present, they will break up. Read after the addition of the saline.
9. Report clumping as positive and lack of clumping as negative.

For results possible, see Table 327.

Tube method for Hr factors using ficinated cells

The test for the **hr′** and **hr″** factors in blood cells employs the rapid ficinating technic (Wiener).

1. Prepare a cell suspension in saline in the usual manner (p. 1463), wash twice with saline, remove the supernatant fluid after the last centrifugation, and resuspend the cells in saline to a 3-5% concentration.
2. Place 2 drops of this suspension in each of 2 Kahn tubes.
3. Add 1 drop of diluted ficin (1% ficin diluted 1:5, p. 1463) to each tube.
4. Mix thoroughly and place in a water bath at 37° C. for 15 min.
5. Mix again, wash once in saline, centrifuge, and remove and discard the supernatant fluid.
6. Add 1 drop of anti-**hr′** agglutinating (tube) serum to the first tube and 1 drop of anti-**hr″** agglutinating (tube) serum to the second tube.
7. Mix thoroughly and place the tubes in a water bath at 37° C. for 30 min.
8. Read reactions by tilting the tubes and observing for clumping or lack of clumping. A pear-shaped sediment when the tube is tilted shows lack of agglutination. Gently dislodge the sediment and examine under a scanning lens of a microscope or with a hand lens.

If the anti-**hr′** serum causes agglutination of the cells, **hr′** factor is present.

If the anti-**hr″** serum causes agglutination of the cells, **hr″** factor is present.

Table 327. *Possible results in Hr slide testing*

*Anti-**hr′** serum plus whole blood*	*Anti-**hr″** serum plus whole blood*	*Conclusions*	*Second half of phenotype symbol*
–	–	**hr′** and **hr″** negative	$\mathrm{rh_y}$ or $\mathrm{Rh_z}$
+	–	**hr″** negative, **hr′** positive	rh″ or Rh_2
–	+	**hr′** negative, **hr″** positive	rh′ or Rh_1
+	+	**hr′** and **hr″** positive	rh or Rh_0

If the cells do not agglutinate in either serum, neither **hr′** nor **hr″** factor is present. Refer to Table 327.

Tube test for Hr factors using untreated cells

1. Prepare 1 tube for each antiserum.
2. Place 1 drop of 2 or 3% saline suspension of unknown washed cells in each tube.
3. Add 1 drop of anti-**hr′** agglutinating (tube) serum to one tube and 1 drop of anti-**hr″** agglutinating serum to the other tube.
4. Mix the contents of each tube separately and incubate in a water bath at 37° C. for 1 hr.
5. Read the sediment in the bottoms of the tubes by slanting the racks; then read over a lighted box and under a scanning lens of a microscope.
6. Report clumping as positive and lack of clumping as negative.

If the cells clump in anti-**hr′** serum, the blood is **hr′** positive. If they fail to clump with anti-**hr′** serum, the blood is **hr′**-negative.

Clumping with anti-**hr″** serum means the blood is **hr″** positive, whereas lack of clumping in this antiserum means that the blood is **hr″** negative. Refer to Table 327.

It is not necessary to make Hr tests on Rh-negative blood since it will be **hr′** and **hr″** positive. Similarly, it is usually unnecessary to test blood containing **rh′** and not **rh″** for the **hr″** factor, except in cases in which an examination for $\mathbf{hr}^{S}$ is being made.

If a blood is **rh″** positive and **rh′** negative, it is not necessary to test for **hr′** because the reaction will be positive. Since the anti-Hr sera are in short supply, they should not be wasted by making unnecessary tests. Exceptions exist. See $\overline{\text{Rh}}_0$ and $\overline{\overline{\text{Rh}}}_0$.

To determine phenotypes in the Rh-Hr system, consult Table 314, p. 1495.

DETECTION OF OTHER Rh-Hr FACTORS IN CELLS

Test for Rh^{A}, Rh^{B}, Rh^{C}, Rh^{D} and their variants in blood cells—Wiener–Unger

1. Prepare a 1% solution of ficin in phosphate buffer as directed on p. 1463.
2. Dilute the ficin 1:5 (1 part ficin + 4 parts saline). **CAUTION: Ficin is a potent proteolytic enzyme. Therefore, do not allow the powder to come into contact with the skin or eyes. If the powder should get onto the skin, wash under running water for a few seconds.**
3. Prepare a 3-5% suspension of blood cells to be tested, wash twice with saline, and resuspend to a 3-5% suspension.
4. Transfer 1 drop of washed cells to each of 4 small Kahn tubes (7-8 mm. inside diameter).
5. Add 1 drop of diluted ficin solution to the cell suspension and mix.
6. Place in a water bath at 37° C. for exactly 15 min.
7. Wash once with saline to stop the action of the enzyme and remove and discard the supernatant fluid. This leaves the ficinated red blood cells in the tubes suspended in 1 drop of saline.
8. Add 1 drop of the antiserum, anti-$\mathbf{Rh}^{A}$, anti-$\mathbf{Rh}^{B}$, anti-$\mathbf{Rh}^{C}$, or anti-$\mathbf{Rh}^{D}$, to their respective tubes and mix.
9. Place in a water bath at 37° C. for 1 hr.
10. Read by observing the sediment in the bottom of the tubes in the usual manner. If the cells have not clumped, they will slowly flow down the tube in a pear-shaped pattern. If they have clumped, they retain their round shape or occasionally the top will fold over. After taking readings from the bottom of the tube, confirm by reading under a scanning lens of a microscope after gently dislodging the sediment.

If the cells have clumped, they contain $\mathbf{Rh}^{A}$ (or any of the other antigens, depending on the specificity of the antiserum) as well as $\mathbf{Rh}_0$. Such cells are simply designated Rh_0 (Rh). If any test is negative, the missing factor is indicated by the use of the appropriate small letter, for example, Rh^{a}, Rh^{b}, etc.

Test for rh^{w} factor in blood cells

Tests for $\mathbf{rh}^{w}$ are made only on cells containing factor **rh′** (Rh_1, rh′, Rh_z, $\mathfrak{R}\text{h}_1$, $\mathfrak{R}\text{h}_z$, and rh_y).

1. Wash once with saline and resuspend to a 2 or 3% concentration in saline.
2. Place 1 drop of saline-suspended cells in a small tube (inside diameter 7 mm.).
3. Add 1 drop of anti-$\mathbf{rh}^{w}$ serum and mix.
4. Place in a water bath at 37° C. for 1 hr.
5. Centrifuge at 500 r.p.m. for 1 min.
6. Gently dislodge the sediment and read macroscopically and under a scanning lens of a microscope. Report clumping as positive and lack of clumping as negative.
7. At the same time run controls, using cells positive for the $\mathbf{rh}^{w}$ factor.

If the unknown cells are clumped by the anti-$\mathbf{rh}^{w}$ serum, they contain the $\mathbf{rh}^{w}$ factor.

If the unknown cells fail to clump in the anti-$\mathbf{rh}^{w}$ serum, they do not contain the factor.

The distinction between positive and negative reactions is generally clear-cut. If desired, centrifuge for 1 min. at 500 r.p.m. and read again.

Because the anti-$\mathbf{rh}^{w}$ sera that are now available are of low titer, it is not recommended that this test be made in medicolegal cases.

Chapter 76

Detection of anti-Rh in blood serum (sensitivity tests)

ADDINE G. ERSKINE

As already pointed out, 2 distinct types of antibody might be formed in blood serum as a result of isosensitization with the Rh agglutinogen: (1) an Rh agglutinating antibody, which clumps cells when they are suspended either in saline or in a high protein medium, and (2) an Rh conglutinating antibody, which coats the cells without clumping them. These are called blocking antibodies because of their in vitro action of preventing agglutination. These antibodies are described on p. 1512. When the cells are suspended in their own plasma or in a fortified protein medium, this conglutinating antibody will cause them to clump. Bovine serum albumin, antihuman globulin, and other media will activate the blocking antibodies. Serum albumin also has the power to cause the blocking antibody to bring about clumping.[145] The mechanism by which serum albumin fractions exert their effects is not entirely clear.

BLOCKING ANTIBODY AND THE PROZONE PHENOMENON IN HUMAN ANTI-Rh SERUM

Wiener and Peters[146] showed that sensitization of Rh-negative individuals against the Rh factor can often be detected by in vitro tests for anti-Rh agglutinins in the serum. However, it was found[133, 147] that there were many Rh-negative patients, strongly sensitized to the Rh factor, whose plasma did not contain demonstrable anti-Rh agglutinins. These patients either had intragroup hemolytic transfusion reactions or had delivered babies with erythroblastosis fetalis.

In searching for an explanation of the lack of in vitro agglutination by these sera, Wiener mixed the sera with group O Rh-positive cells and later added known anti-Rh serum to the same test cells. The cells were not agglutinated by the known anti-Rh serum after they had been in contact with the patients' sera. He concluded that the action of the active agglutinin in the added serum had been blocked, and designated such antibodies as were present in these patients' sera as blocking antibodies. Fig. 494 is a diagrammatic representation of the difference between Rh agglutination and blocking reactions.

Tests were carried out on a number of patients with erythroblastotic infants on whom the usual tests for anti-Rh agglutinins were unsuccessful, and in most of the cases clear-cut blocking reactions were obtained, demonstrating the presence of a special type of Rh antibody. The reaction is specific in that it is obtained only with sera from Rh-negative individuals sensitized by the Rh factor and not with sera from Rh-positive or Rh-negative individuals not sensitized by the Rh factor.

A number of human anti-Rh sera have been obtained which exhibit a marked "prozone" effect.[148, 149] Taylor et al.[148] attempted to explain the behavior of such sera on the basis of optimal proportions of antigen and antibody. This does not explain why the phenomenon does not occur more frequently. Wiener's experiments indicate that such prozone phenomena are due to the presence in sera of a mixture of blocking and agglutinating antibodies, the agglutinating antibodies being of a higher titer.

Those sera that exhibit the prozone effect cannot be used as diagnostic reagents unless the agglutinin is far more active than the blocking antibody. Experiments seem to indicate that some of these sera can be absorbed

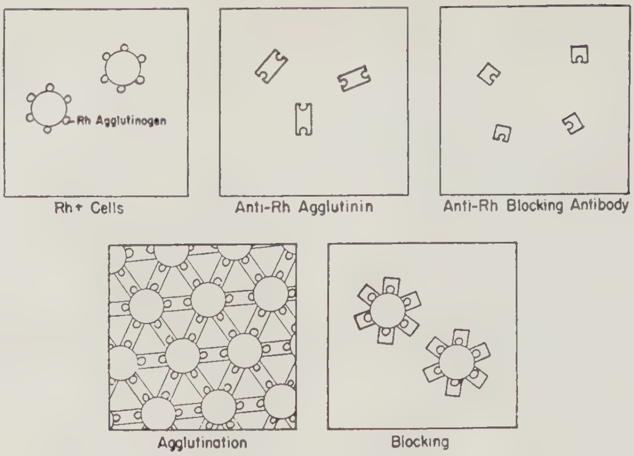

Fig. 494. Diagrammatic representation of Rh agglutination and blocking reactions. (From Wiener, A. S.: Am. J. Clin. Path. 15:106, 1945.)

with Rh-positive blood, so that after the blocking antibody is specifically removed the treated serum becomes a useful diagnostic reagent. From a practical standpoint, the significance of these findings lies in the fact that the characteristics of the agglutinating antibody and the blocking antibody are sufficiently clear-cut, so that, for example, there is no difficulty in the use of an anti-Rh serum which contains an anti-**rh′** agglutinin and also an anti-**Rh**$_0$ blocking antibody. In fact, Wiener has prepared anti-**rh′** reagents from anti-**Rh**$'_0$ sera by adding anti-**Rh**$_0$ blocking antibody.

It is often necessary to determine the presence in the patient's blood serum of Rh-Hr and other antibodies. Three tests are commonly made: (1) the agglutination test, (2) the blocking test, and (3) the conglutination test. These tests are made simultaneously and are referred to as "sensitivity" tests.

Sensitivity tests for Rh-Hr and other antibodies should be made of the sera of both the patient and the donors. These should be routine tests even if the cross-matching tests are compatible. If antibodies are present, they could be responsible for posttransfusion intragroup reactions should the patient be transfused with cells containing antigens for which these antibodies are specific. As an example, if the patient has anti-**hr′** and is transfused with blood having the **hr′** factor, a transfusion reaction might result. For further discussion refer to p. 1507.

A number of biological houses have made available to the public preserved cells containing all the rare antigens. These are called "Hemantigen,"* Type-A-Cells, and Panel Cells* or they are known by other names. Included in these specially selected human red cells are all the Rh-Hr antigens, and, in addition, **K-k**, **Fy**^{a} and **Fy**^{b}, **Le**(a+) and **Le**(b+), **Jk**^{a} and **Jk**^{b}, **M**, **N**, **S**, **s**, **P**, **Lu**^{b}, **Yt**^{a}, **U**, **Vel**, and others. By use of these cell panels one can detect the presence of antibodies without actually identifying them. If the exact identification of an antibody must be known, then a whole battery of specific antigens must be at hand, and the serum must be tested against each antigen.

The tests for antibody content of unknown serum is made as an aid in the management of pregnant women, as a means of predicting and preparing for the treatment of an erythroblastotic infant and as an aid in the search for prospective donors of Rh-Hr antibodies. In the method that follows, tests are limited to detection of the 5 principal Rh-Hr antibodies: anti-**Rh**$_0$, anti-**rh′**, anti-**rh″**, anti-**hr′**, and anti-**hr″**. Group O cells are used because they contain neither A nor B agglutinogen. Type Rh$_z$Rh$_0$ cells are used because they react positively with all 5 standard Rh-Hr antibodies.

Tests for Rh-Hr antibodies in serum (Fig. 483)

Have at hand group O Rh$_z$Rh$_0$† cells. Group O is used because the unknown serum might contain anti-**A** or anti-**B** agglutinins which would react with cells derived from groups A, B, or AB but not with group O. Type Rh$_z$Rh$_0$ cells are selected because they react with anti-**rh**′, anti-**rh**″, anti-**Rh**$_o$, anti-**hr**′, and anti-**hr**″ antibodies.

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*Knickerbocker Laboratories, New York, N. Y.

*Certified Blood Donors Service, Inc., Jamaica, L. I., N. Y.

†The symbol Rh$_z$Rh$_0$ includes both Rh$_z$rh and Rh$_1$Rh$_2$, just as the symbol A includes both A$_1$ and A$_2$.

1. Prepare 2 cell suspensions of group O Rh$_z$Rh$_o$ cells:
   (a) Untreated and saline-suspended, 3% concentration.
   (b) Trypsinated or ficinated, 5% concentration.
2. Use 2 tubes, 7.5 cm. × 8 mm., for each serum tested (p. 1463).
3. Place 1 drop of untreated saline-suspended group O Rh$_z$Rh$_o$ cells in the first tube and 1 drop of group O Rh$_z$Rh$_o$ trypsinated or ficinated cells in the second tube.
4. Add 1 drop of the serum to be tested to each tube and shake.
5. Incubate in a water bath at 37° C. for 30 min.
6. Tilt the tubes back and forth gently over a lighted viewing box and observe for clumping or lack of clumping. Read under a scanning lens of a microscope.

If any tube appears to have clumps, the individual is sensitized and has antibodies against the Rh-Hr factors. If all tubes appear to be negative, proceed with the antiglobulin test.

Antiglobulin tests (indirect Coombs test)

Antiglobulin tests are made on negative-reacting untreated and trypsinated cells, saline-suspended.

1. Add saline to each tube to full capacity and mix thoroughly.
2. Centrifuge to pack and pour off the supernatant saline.
3. Shake and add more saline, centrifuge, remove saline, and shake again. This is the second washing.
4. Add more saline, mix, and centrifuge to pack the cells firmly. Pour off the supernatant saline and strike the lip of the tube upside down against a gauze pad to remove all traces of saline.
5. Shake to dislodge the cells.
6. Incubate at 37° C. for 5 min.
7. Add a drop of antihuman globulin serum (Coombs serum) to each tube.
8. Mix and centrifuge at 500-1000 r.p.m. for 2 min.
9. Gently dislodge the sediment, tip back and forth, and read under a scanning lens of a microscope.

Note: Unger performs Coombs tests on all negatively reacting specimens using ficinated cells. This is the **Unger test,** also called the **ficinated cell-antiglobulin test,** and is very important.

If clumping occurs, the individual is sensitized and has an antibody in the serum specific for a factor present in the clumped cells. Its specificity remains to be determined. If clumping occurs with saline-suspended cells without the addition of antiglobulin serum, there are agglutinating or bivalent antibodies in the serum. If it occurs with enzyme-treated cells or upon the addition of antiglobulin serum but not with saline-suspended cells, univalent (or blocking) antibodies are present.

If any clumping occurs, this must be reported. If it occurs with the donor's serum, reject the donor. If it occurs with the patient's serum, report to the director of the laboratory and prepare for identification and titration of antibodies.

If no group O Rh$_z$Rh$_0$ cells are available, use groups O Rh$_1$, O Rh$_2$, and O rh untreated, saline-suspended cells, also trypsinated cells. Make Coombs tests on negatively reacting specimens in tubes containing both untreated and trypsinated cells (Unger test). Unger also makes antiglobulin tests when ficinated cells have been used and negative results are obtained.

Test for Rh-Hr and rare antibodies in serum—sensitivity testing using cell panels

The same method may be carried out (see above), except that cells containing all the antigens are used. These are available commercially either untreated or as enzyme-treated cells. Untreated and enzyme-treated specific antigen cells are also available for cases in which it is necessary to identify the antibody. The method of using is the same as that above or in the following procedure.

Test for antibodies in serum using cell panels

In order to make complete tests for antibodies in an unknown serum, a suitable panel of known blood cells is necessary. These are cell suspensions containing known antigens and are derived from group O individuals. Such cells are available from a number of sources.*

1. Use a small tube (7 mm. inside diameter) for each group of antigens, and label the tubes with the identification of the antigens being tested.
2. Place 2 drops of the antigen cells in the properly labeled tube.
3. Add 2 drops of serum containing the unknown antibody.
4. Mix and centrifuge immediately for 1 min. at 1000 r.p.m.
5. Take a preliminary reading, recording clumping as a positive reaction and absence of clumping as a negative reaction.
6. Incubate the tubes giving negative reactions for 1 hr. at 37° C. If a cold agglutinin is suspected, incubate at 4° C.
7. Centrifuge the tubes for 1 min. at 1000 r.p.m. and read.

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*Certified Blood Donor Service, Inc., Jamaica, L. I., N. Y.; Knickerbocker Foundation, Inc., New York, N. Y.

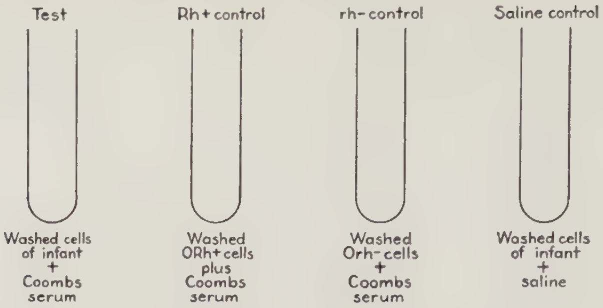
Test
Rh+ control
rh− control
Saline control
Washed cells of infant + Coombs serum
Washed ORh+ cells plus Coombs serum
Washed Orh− cells + Coombs serum
Washed cells of infant + saline

Fig. 495. Coombs test on cord or infant's blood (direct Coombs test).

8. Use the tubes that show a negative reaction. Wash the contents 3 times with saline, remove all traces of saline after the last washing, and add 1 drop of antiglobulin (Coombs) serum.
9. Mix, recentrifuge, and read. Agglutination of the cells in this step indicates that univalent antibodies are present in the serum.

Conglutination test for sensitivity to Rh-Hr factors—Wiener

The conglutination test requires that the cells be suspended in their own plasma or plasma-albumin mixture. To prepare the cells, make a 5% suspension of group O Rh_zRh_o (or other cells) in their own serum or plasma or in plasma-albumin mixture and carry out the test against the unknown serum as directed on pp. 1522 and 1523.

Indirect test for Rh_o blocking antibodies—Wiener

Procedure: Carry out the test in small Kahn test tubes. Use 1 drop of the patient's serum mixed with 1 drop of saline-suspended group O Rh-positive cells (Rh_o, Rh_1, Rh_2, or Rh_z).

1. Incubate for 1 hr. in a water bath at 37° C.
2. Centrifuge at 1000 r.p.m. for 1 min.
3. Read under a scanning lens.

If clumping occurs, Rh_o agglutinating antibodies are present in the unknown serum.

If the reaction is negative, centrifuge, remove the supernatant fluid, and add to the same tube 1 drop of known anti-**Rh**$_o$ agglutinating serum.

Mix and again place in a water bath at 37° C. for 1 hr., and read. Then centrifuge for 1 min. at 1000 r.p.m., shake gently, and take a second reading.

If agglutination now occurs, the patient's blood serum contains neither agglutinating nor blocking **Rh**$_o$ antibodies.

If no agglutination occurs, the patient's serum contains **Rh**$_o$ blocking antibodies; otherwise the known Rh-positive cells would have reacted with the added known anti-**Rh**$_o$ serum.

This same test may be used to detect other blocking antibodies, using specific cells in the original test and a specific antiserum when the initial reaction is negative.

Direct Coombs test—direct antiglobulin test on cord blood and infant's blood (for univalent antibodies derived from the mother)—Wiener[150-152]

The antiglobulin or direct Coombs test is used routinely whenever antibodies that react to antihuman globulin addition are to be detected. These are the univalent or blocking antibodies. These antibodies are also detected by the blocking, conglutination, and enzyme technics.

As Wiener first pointed out, the direct antiglobulin test can be used to detect the presence of univalent (blocking) antibodies coating the red cells of erythroblastotic infants after birth.* These univalent antibodies, when present, have been derived from the isosensitized mother and are considered the cause of erythroblastosis fetalis. When they are found, the baby will generally develop clinical signs of erythroblastosis fetalis if left untreated, and an exchange transfusion should be performed, using Rh-negative donor blood (see pp. 1537ff).

Procedure:

1. Wash the baby's blood cells 3 times with saline. After the last washing and centrifugation, turn the tube upside down and strike the lip on a gauze pad to remove all the saline.
2. Resuspend the cells to approximately 2% suspension in saline.
3. Place 2 drops of washed saline-suspended cells in a scrupulously clean, dry, 7 × 70 mm. tube. See Fig. 495.
4. Add 2 drops of antihuman globulin (Coombs) serum.
5. If the cells have been strongly sensitized by the maternal antibodies, clumping will be-

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*As Wiener pointed out, the coating of the baby's cells can also be demonstrated by the blocking and direct conglutination tests. See Wiener and Sonn[150] and Wiener and Wexler.[151]

come visible almost immediately. Examine over a lighted viewing box.

6. If there is only a weak reaction or if the reaction appears to be negative, allow the tube to stand at room temperature for 15-30 min.
7. Centrifuge at 500 r.p.m. for 2 min.
8. Observe for agglutination over a lighted viewing box and under a scanning lens.

Agglutination means that the infant's cells have been coated with blocking antibodies derived from an isosensitized mother. Lack of agglutination means that the baby's cells are not coated.

Controls: Controls are made in small tubes.

1. Two drops of infant's washed cells plus 2 drops of saline.
2. Two drops of saline suspended Rh-positive cells plus 2 drops of antiglobulin serum.
3. If desired, prepare Rh-positive cells by coating them with known blocking antibodies. Add 2 drops of 2% suspension of Rh-positive saline-suspended cells to 2 drops of known Rh conglutinating (slide) (blocking) serum. Incubate at 37° C. for 30 min. There should be no agglutination. Centrifuge and remove the supernatant saline and wash 3 times with saline. These are the coated cells. Add 2 drops of Coombs serum (antiglobulin). The cells should now clump.
4. Two drops of Rh-negative cells plus 2 drops of antiglobulin serum.

Handle the controls in the same manner as the test.

Tubes 1, 2, and 4 should give negative reactions.

The antihuman globulin serum may give a false negative direct or indirect reaction when the red blood cells are coated with a minimal amount of antibody. In such cases prepare a 1:5 dilution by adding 1 drop of antihuman globulin to 4 drops of saline and use 1 drop in the test.

Antihuman globulin serum (Coombs serum) is prepared by hyperimmunizing rabbits against human serum globulin or whole human serum. Nonspecific antibodies are absorbed from the serum by adding human red cells which have been washed 10 times with saline solution.

Indirect antiglobulin test (Coombs test) for detection of isoimmunization[152]

See Rh-Hr sensitivity testing, pp. 1522 and 1523.

Quantitative direct antiglobulin test—Wiener[153]

This is a test for measuring the degree of coating of red cells by antibodies from an erythroblastotic infant or a patient with hemolytic anemia.

1. Wash the red cells to be tested 4 times with an excess of saline, as for the simple direct antiglobulin test.
2. Titrate the antiglobulin (Coombs) serum in serial dilutions against these washed cells.
3. At the same time titrate the antiglobulin serum in serial dilutions against Rh-positive cells which have been maximally coated by sensitizing them with an anti-Rh conglutinating (slide test) serum having a titer well in excess of 16 units by the albumin-plasma method (p. 1530).
4. Calculate the percentage of coating of the unknown cells by dividing the titer of the antiglobulin serum for these cells by its titer for maximally coated Rh-positive cells and then multiplying by 100.

This method has been applied by Wiener to determine the relative number of antigenic points in the discoplasm for agglutinogens of different blood group systems. It is also applied in babies with erythroblastosis as an additional criterion of severity and in patients having acquired hemolytic anemia, in whom it has been found that the degree of coating is correlated with the clinical manifestations. Thus a rise in the degree of coating may be associated with a hemolytic crisis, whereas a decrease may presage a remission. In disseminated lupus erythematosus there is milder manifestation of autosensitization; this view is supported by the results of the quantitative direct antiglobulin test.

False negative reactions with antiglobulin serum—Coombs

False negative antiglobulin tests result from a number of causes. These may be summarized as follows:

1. Minute traces of serum or plasma in the test cells may result in false negative Coombs tests. All test cells must be thoroughly washed with saline to remove any human protein present. At least 10 vol. saline must be used for each washing, and the cells should be washed at least 3 times.

The glassware must be scrupulously clean since traces of dried blood left in the tubes in many cases will result in false negative antiglobulin tests.

To resuspend the cells after each washing, use either a piece of wax paper or Parafilm over the top of the tube when inverting it, or use a clean cork.

Cells must not be centrifuged excessively since such packed cells prevent removal of small amounts of protein into the saline wash solution.

2. Avoid the use of old cells. Wherever possible, use cells freshly obtained. Do not keep saline-suspended cells more than 24 hr.

3. Avoid using too weak a suspension of cells.

4. Avoid using outdated or deteriorated antiglobulin serum. This is a general precaution which must be taken with every antiserum used in blood grouping.

5. Always run positive and negative controls with each direct antiglobulin test. For such controls $\mathbf{Rh_o}$-positive cells must be coated with anti-$\mathbf{Rh_o}$

conglutinating (slide) serum, washed, and the antiglobulin test run simultaneously with the unknowns.

AGAMMAGLOBULINEMIA

Inhibition test for human globulin, using coated (sensitized) Rh-positive cells—Wiener[154-156]

Agammaglobulinemia was first described as a syndrome by Bruton[157] and later elaborated by Bruton et al.[158] Patients with agammaglobulinemia show complete or almost complete absence of antibodies from their serum, rendering them susceptible to recurrent infections. Their serum lacks the normal blood group isoagglutinins, as well as gamma globulin as detected electrophoretically. The other serum proteins may be within normal limits.

A test for agammaglobulinemia was devised by Wiener,[155] based on the ability of antihuman globulin sera prepared in rabbits to agglutinate human red cells coated with antibodies of human origin, such as Rh antibodies and autoantibodies. Since materials containing human gamma globulin can inhibit the reactions of such antiglobulin sera, this inhibition technic has been applied to detect the presence of human gamma globulin in patient's serum. The test can be performed quantitatively[156] by testing a series of progressively doubled dilutions of the material in question, at the same time carrying out parallel titrations with normal human serum as a control.

The sensitivity of the inhibition test for gamma globulin is inversely related to the titer of the antiglobulin serum used. If a low-titered serum is used, the inhibition titer will be high; the higher the titer of the antiglobulin serum, the lower the gamma globulin inhibition titer. Thus if the test is performed qualitatively only, the diagnosis of agammaglobulinemia could be missed.

Principle: The solution to be tested for the presence of serum globulin is mixed with absorbed antihuman globulin serum (diluted to have a titer of 10-20 units for maximally coated Rh-positive cells) and the mixture allowed to react in a water bath at 37° C. for 1 hr. Then a suspension of Rh-positive cells, coated with **Rh$_0$** univalent antibody, is added, and the mixture is allowed to stand for a second period of incubation, after which the reaction is read. Failure of clumping to occur indicates the presence of human serum globulin in the material being tested. Occurrence of clumping indicates the absence of human serum globulin.

Reagents:

1. Rh-sensitized cells.
   - (a) Prepare a 2% suspension of **Rh$_0$**-positive cells in saline.
   - (b) Wash once with saline solution and resuspend to 2% concentration.
   - (c) Mix with an equal volume of **Rh$_0$** antiserum containing univalent antibodies with a titer of approximately 100 units by the albumin-plasma method (p. 1530).
   - (d) Allow the mixture to react in an incubator or water bath at 37° C. for 45 min.
   - (e) Centrifuge at moderate speed.
   - (f) Discard the supernatant fluid.
   - (g) Wash the sedimented cells 4 times with large volumes of saline.
   - (h) Resuspend sensitized cells to an approximate 2% suspension in saline.
2. Antiglobulin serum.

   Antiglobulin serum is commonly referred to as Coombs serum and is available commercially. It is absorbed according to the requirements of the NIH before being packaged. This is diluted so as to have a titer of 10-20 units for the Rh-sensitized cells.

Procedure:

1. Prepare serial dilutions in saline of the serum to be tested and of normal serum in the same manner as for titrations, as given on p. 1529. Use dilutions of 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:512, etc.
2. Place 1 drop of each dilution in its respective small Kahn tube, properly labeled.
3. Add 1 drop of diluted antihuman globulin serum to each.
4. Incubate at 37° C. in a water bath for 45 min.
5. Add 1 drop of Rh-positive sensitized cells to each tube.
6. Shake the tubes and reincubate for 60-90 min. in a water bath at 37° C.
7. Shake gently and read the reactions macroscopically over a lighted viewing box and under a scanning lens of a microscope.

Results: Normal human adult serum completely inhibits agglutination in dilutions as high as 1:500 on the average, depending on the titer of the antiglobulin serum.

Normal human spinal fluid in Wiener's experiments inhibited the antiglobulin serum from reacting in dilutions of approximately 1:2 or 1:4, but this was expected because normal spinal fluid contains only about 30 mg.% protein in contrast to the 7 gm. protein found in normal human serum. Saliva showed inhibition ability to a slight degree; umbilical cord serum inhibited antiglobulin serum to the same titer as adult serum. Serum from horse, ox, and rabbit did not inhibit antiglobulin serum, and rhesus monkey serum weakened its reaction.

In cases of congenital agammaglobulinemia, inhibition titers of about $\frac{1}{50}$ the normal titer were obtained. This indicates the presence of only 20-30 mg. of gamma globulin/100 ml.

serum in such individuals rather than a complete absence of gamma globulin. Following treatment with gamma globulin there is a corresponding increase in the inhibition titer.

TITRATION OF Rh ANTIBODIES IN SERUM AND IDENTIFICATION OF SPECIFICITY—UNGER

In the case of Rh-negative pregnant women who have had previous transfusions or pregnancies, Rh antibody titrations should be made before the third month to determine whether antibody is being carried into their present pregnancies. When antibody is demonstrated, titrations should be made at monthly intervals until the seventh month and at biweekly intervals thereafter until the last month, when they should be made weekly. When the titrations at the third month do not demonstrate Rh antibody, no further studies need be made until the seventh month.

The appearance of antibody or a rising titer when antibody is demonstrated at the third month proves that immunity is stimulated and that the fetus is Rh positive. An unchanging or falling titer proves that immunity has not been stimulated; the fetus may be Rh positive or Rh negative. When there is a rise in titer, on the other hand, the fetus is invariably Rh positive.

Rh antibody titrations are made on bloods of all Rh-negative sensitized patients and on all other bloods as ordered. For example, serum from Rh-negative patients is titrated against (1) group O type Rh_1Rh_1 (if not available, use type Rh_1rh), (2) group O type Rh_2rh, (3) group O type rh′rh, (4) group O type rh″ rh, and (5) group O type rh (triple Rh negative). The serum of Rh-positive patients sensitized against specific Rh factors may also be titrated against these specific cells. Several biological houses have specific cells for titration purposes; if the antibody must be identified and its concentration determined, specific antigen cells are used in the titration.

If the patient lacks $\mathbf{Rh_0}$ but has **rh′**, the blood is not titrated against **rh′**. If the patient is sensitized against the Hr factors, the serum is titrated against Rh-negative blood, which contains **hr′** and **hr″**, and Hr-negative blood is used as a control instead of Rh-negative blood (Rh-negative blood is Hr positive).

Six titrations are run simultaneously in some instances, but a simplified method, p. 1532, is recommended for routine practice:

1. Saline agglutination titer for bivalent antibodies.
2. Trypsinated cell titer.
3. Indirect blocking antibody titer for univalent antibodies.
4. Antihuman globulin titer for univalent antibodies.
5. Albumin-plasma titer for univalent antibodies.
6. Trypsinated cell-antihuman globulin titer for univalent antibodies.

The serial dilutions used are 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512. Additional dilutions are made if the titer is 512 or higher.

If the sensitivity tests made before the titration indicate a weak antibody titer, some of the tubes may be omitted.

Three racks of 10 holes and 4 rows are needed for the titration proper (2 for saline-suspended cells and 1 for trypsinated cells), and one rack is used to make the serum dilutions. A rack with 2 rows of holes may be used to make the dilutions. The technic of handling dilutions is extremely important; no traces of serum or of the various dilutions must be carried from one tube to another, since that would erroneously increase the titer. The same size drop of each dilution must be used.

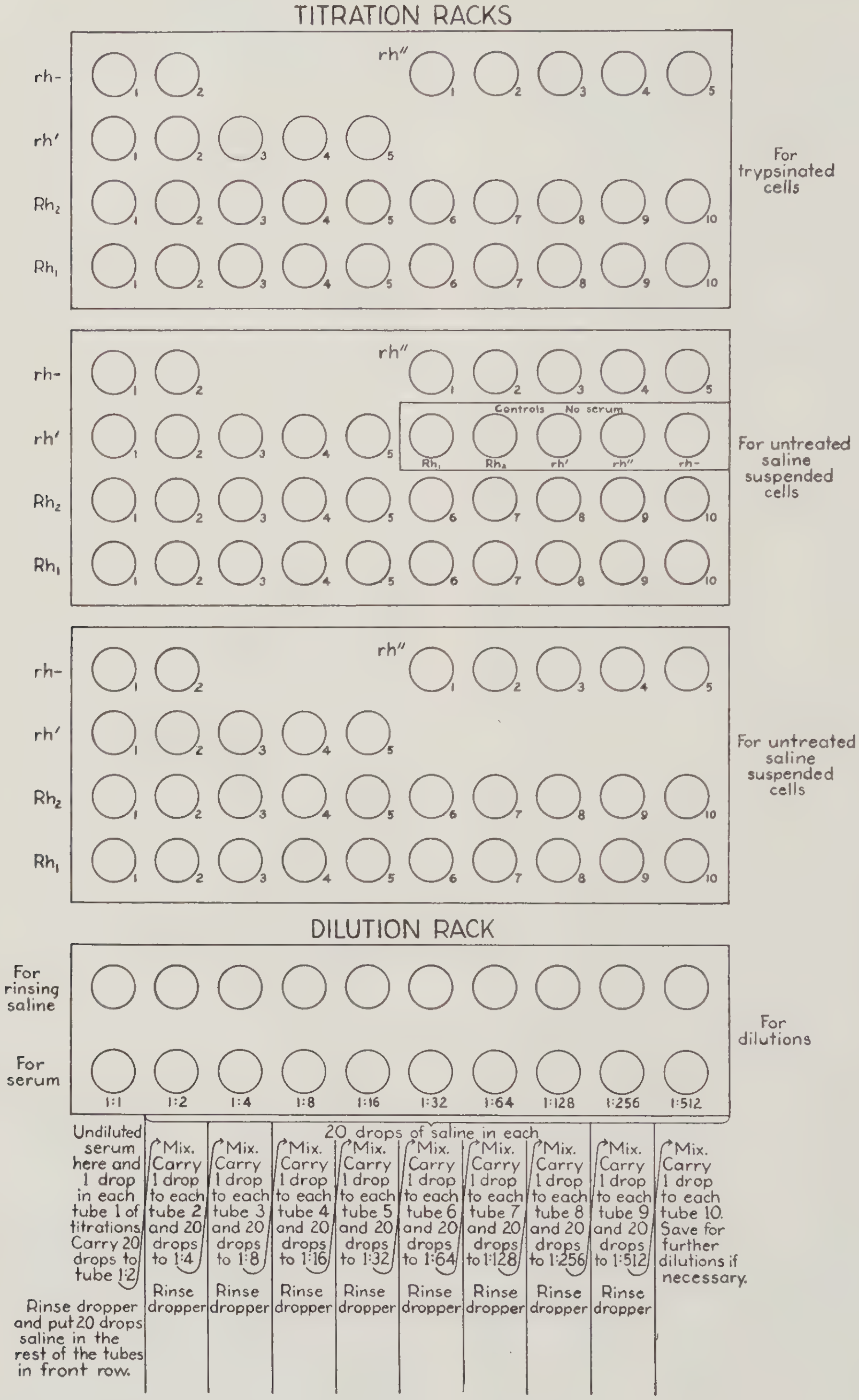
The cells needed are listed above. One set must be untreated and suspended in saline to a 2 or 3% concentration; they are washed once and resuspended in saline. Trypsinated cells are prepared as usual (p. 1463), and resuspended to 3-5% in saline.

Albumin-plasma mixture:

1. Pool a number of groups O, A, and B plasmas.
2. Centrifuge and remove the clear plasma.
3. Measure 8 drops of 30% bovine albumin into a tube.
4. Rinse the dropper with the pooled plasma, discarding each dropperful of rinsing plasma.
5. Repeat this 2 or 3 times, depending on how much plasma is available. This is to remove the bovine albumin from the dropper without admitting any water or saline.
6. Using the same dropper, add 32 drops of the pooled plasma, giving a dilution of 1 part albumin to each 4 parts pooled plasma.
7. Mix the albumin-plasma mixture in and out of the dropper before using.

For the indirect blocking test, anti-$\mathbf{Rh_0}$ saline agglutinating tube test serum will be needed.

When the titrations have been prepared, the first row of tubes in the titration racks will contain serially diluted serum plus group O Rh_1Rh_1 cells; the second row, the serially


TITRATION RACKS
rh-
rh′
Rh2
Rh1
rh″
1
2
3
4
5
6
7
8
9
10
For trypsinated cells
Controls No serum
Rh1
Rh2
rh′
rh″
rh-
For untreated saline suspended cells
For untreated saline suspended cells
DILUTION RACK
For rinsing saline
For serum
For dilutions
1:1
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
20 drops of saline in each

Undiluted serum here and 1 drop in each tube 1 of titrations. Carry 20 drops to tube 1:2

Rinse dropper and put 20 drops saline in the rest of the tubes in front row.

Mix. Carry 1 drop to each tube 2 and 20 drops to 1:4. Rinse dropper

Mix. Carry 1 drop to each tube 3 and 20 drops to 1:8. Rinse dropper

Mix. Carry 1 drop to each tube 4 and 20 drops to 1:16. Rinse dropper

Mix. Carry 1 drop to each tube 5 and 20 drops to 1:32. Rinse dropper

Mix. Carry 1 drop to each tube 6 and 20 drops to 1:64. Rinse dropper

Mix. Carry 1 drop to each tube 7 and 20 drops to 1:128. Rinse dropper

Mix. Carry 1 drop to each tube 8 and 20 drops to 1:256. Rinse dropper

Mix. Carry 1 drop to each tube 9 and 20 drops to 1:512. Rinse dropper

Mix. Carry 1 drop to each tube 10. Save for further dilutions if necessary.

Fig. 496. Set-up for full titrations for Rh antibodies. (After Unger.)

diluted serum plus group O Rh_2rh cells; the third row, group O rh′rh cells (5 tubes unless a high titer is anticipated); and for the fourth row, the first 2 tubes will contain serially diluted serum and group O Rh-negative cells, and serially diluted serum plus group O rh″rh cells will be in the second half of the fourth row.

Rack 2 is used for the controls on the various cells suspended in saline, plus the albumin-plasma mixture, to make sure that these cells do not agglutinate in the albumin-plasma mixture without the patient's serum.

One drop of each serum dilution is used for each drop of cells.

Serial dilution of serum and technic of titration (Fig. 496): Dilutions and titrations are made at the same time so that the same dropper can be used throughout the procedure, ensuring uniform drops in each tube.

1. Rack up the 3 racks of tubes, 7.5 cm. × 8 mm., for the titrations. Be sure to label at least the first tube in each row. Use a different color wax pencil for the trypsinated cells titrations. When antiglobulin tests are carried out, each tube must be labeled.
2. Rack up a set of Kahn tubes in 2 rows of 10 tubes each for the serum dilutions.
3. Use serum, **not** plasma, for the titration whenever possible.
4. Place the serum in tube 1 of the front row of the dilution rack. Leave the other 9 tubes in that row empty. Label the tubes 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512, respectively.
5. Fill all the back row of dilution tubes with saline. There are now 2 rows of Kahn tubes, the first row empty except for serum in the first tube and the second row of tubes filled with saline.
6. Put 1 drop of undiluted serum into each first tube of each row in the titration.
7. Using the same dropper, carry 20 drops from the first tube of serum to the second tube in the front row of the dilution rack.
8. Rinse the dropper with all the saline in tube 1 of the back row of dilution tubes and remove the discarded saline tube from the rack. Wipe off the excess saline with a gauze pad.
9. Put 20 drops of clean saline into each tube of the dilution rack, front row, beginning with tube 2 and using the same dropper, which has been rinsed with saline, held at the same angle.
10. Mix the contents of tube 2 thoroughly in and out of the dropper, avoiding bubbles.
11. Transfer a drop of this dilution into each second tube of the titrations, holding the pipet at the same angle throughout and being careful not to add bubbles, for these are not full drops.
12. Carry 20 drops of diluted serum from tube 2 to tube 3 of the dilution rack.
13. Rinse the dropper with the saline in the tube behind tube 2, using all the saline, and wiping off the excess on a gauze pad.
14. Mix contents of tube 3 thoroughly, in and out of this same dropper, carry a drop to each tube 3 in the titrations, and carry 20 drops to tube 4 in the dilution rack.
15. Rinse the dropper with the saline behind tube 3 and continue in the same manner as above until all dilutions have been made and all the tubes in the titration racks have received their respective diluted serum, 1 drop each.
16. Do not put any of the diluted serum in the control tubes that are run on the cell suspensions which are to receive albumin-plasma mixture.

Adding untreated saline-suspended cells:

1. Add 1 drop of untreated saline-suspended group O Rh_1Rh_1 cells to each tube in the front row of titration racks 1 and 2.
2. Add 1 drop of group O Rh_2rh untreated saline-suspended cells to each tube of the second row in titration racks 1 and 2.
3. Add 1 drop of group O rh′rh cells untreated and saline suspended into each tube of the third row of titration racks 1 and 2.
4. Add 1 drop of group O Rh-negative cells, untreated and saline suspended, into the tubes marked for these cells, the first 2 or 3 in row 4 of the titration racks 1 and 2. If full titrations are run, there will be 10, not 2 or 3, of these tubes.
5. Add 1 drop of group O rh″rh untreated saline-suspended cells to each of the tubes marked for these cells (in the right-hand side of row 4 of racks 1 and 2 of the titrations). Full titrations may be run on these if desired, requiring 10 tubes, which would mean 5 rows of tubes for racks 1, 2, and 3, instead of 4 rows.
6. Prepare the control cell suspension tubes in rack 2, or else use a separate rack. Add 1 drop of each cell type to its respective tube. These will not receive any serum.

Preparation for trypsinated cell titrations:

1. In rack 3 for the titrations add 1 drop of group O type Rh_1Rh_1 trypsinated cells to each of the front row of tubes.
2. Add 1 drop of group O type Rh_2rh trypsinated cells to the second row of tubes.
3. Add 1 drop of group O type rh′rh trypsinated cells to the third row of tubes.
4. Add 1 drop of group O type rh″rh trypsinated cells to the fourth row of tubes.
5. Add 1 drop of group O type rh trypsinated cells in the tubes marked for these cells in row 4.
6. Incubate all 3 racks, after mixing each tube, for 1 hr. in a water bath at 37° C. The bath should be covered with a white cloth to keep dust out of the tubes.
7. At the end of the hour examine the bottoms of the tubes but do not disturb rack 2, which is exactly like rack 1 up to this point (except for the controls). It will be used later for the albumin-plasma mixture. It must stand undisturbed.

Saline agglutination titer

Read the result in each tube of **titration rack** 1 under a scanning lens of a microscope, tilting the tube back and forth to resuspend the cells gently. The weakest dilution with a positive reaction is the titer. This gives the **saline agglutinating titer.**

Albumin-plasma titer—Wiener

1. Using a dropper with a fine capillary, remove all the saline from each tube in rack 2, beginning with the weakest dilution and working back to the strongest. Do not introduce any serum into the control tubes, which must be handled first. In other words, remove the saline from the control tubes, then begin with tubes 10 and remove the saline from each tube 10, then from each tube 9, then each tube 8, etc., down to tube 1.
2. Prepare the albumin-plasma mixture as directed (p. 1527), and while the dropper (this is a second dropper) contains the mixture, add 1 large drop of the mixture to each tube in titration rack 2, including the controls.
3. Shake the rack thoroughly. Incubate at 37° C. in a water bath for 1 hr.
4. At the end of the hour read under a scanning lens of the microscope, after looking at the bottoms of the tubes. Record clumping as positive and no clumping as negative.

Always read the controls first and use the negative control tubes for comparison when not sure of a negative reading.

The weakest dilution with a positive reaction is the **albumin-plasma titer.**

Indirect blocking antibody titer—Wiener

1. Using titration rack 1, remove the saline from each tube in the front row of tubes where there is a negative reaction. If the reaction is positive, this test is omitted. This test is for the indirect blocking reaction. Only the first row of tubes is used.
2. Add 1 drop of anti-**Rh**$_0$ saline agglutinating serum to each tube which shows a negative reaction, and **only to row 1 of the first rack.** This detects the **Rh**$_0$ blocking antibody.
3. Shake and replace the tubes in a water bath at 37° C. for 1 hr.
4. At the end of the hour read under a scanning lens, and record. The last tube showing a **negative** reaction is the titer.

Explanation: In the first hour the patient's serum was mixed with known Rh-positive cells. If these cells did not agglutinate, either the patient's serum did **not** contain bivalent (saline-agglutinating) antibodies or else it **did** contain univalent (blocking) antibodies, which simply coated the cells but did not agglutinate them. Known anti-**Rh**$_0$ agglutinating serum was then added, which has the ability to agglutinate Rh-positive cells. Since these Rh-positive cells did not agglutinate, they must have been coated with blocking antibody derived from the patient's serum, which prevented their clumping when the known anti-**Rh**$_0$ serum was added. If they had clumped, that would indicate that there were no blocking antibodies in the patient's serum.

This gives the **indirect blocking titer.**

Antihuman globulin titer—Coombs

1. Using rack 1, add saline to each negative tube in rows 2, 3, and 4 after shaking. Fill the tubes about ¾ full.
2. Centrifuge and pour off the supernatant fluid, catching the last drop on a gauze pad.
3. Shake each tube, add saline again, and again centrifuge. This is the second washing. Remove the saline, replace with fresh saline, centrifuge, and remove saline, this time tapping the tubes hard against a gauze pad to remove all traces of saline. Take care not to lose the cells. This is the third washing.
4. Shake the tubes and add a drop of Coombs serum (antiglobulin) to each tube.
5. Centrifuge for 2 min. at 500-1000 r.p.m. without incubation.
6. Read all tubes under a scanning lens of a microscope and report clumping as positive and lack of clumping as negative.

Read the controls first for comparison. The weakest dilution with a positive reaction is the **antiglobulin titer.**

This completes the tests on saline-suspended untreated cells.

Trypsinated cell titer—Morton-Pickles

At the end of the hour's incubation of rack 3 containing the trypsinated cells, read under a scanning lens. These tubes are handled not quite so gently as the untreated cells. Read the negative reactions first for comparison; then read the other tubes. Record clumping as positive and lack of clumping as negative. The weakest dilution with a positive reaction is the **trypsinated cell titer.**

Trypsinated cell-antiglobulin titer—Unger

1. Use all the tubes in rack 3 which give a **negative** reaction.
2. Wash 3 times with saline as directed above in the other tests.
3. After the last washing and packing, remove all the saline by tapping hard against a gauze pad, resuspend the cells, and add a drop of Coombs serum to each tube.
4. Centrifuge for 2 min. at 500-1000 r.p.m. and read the reactions under a scanning lens.

The weakest dilution with a positive reaction is the **trypsinated cell-antiglobulin titer.**

Reactions are recorded by Unger as ++s, ++, ++w, +s, +, +w, ±, and −.

++s means a solid clump which does not break up when being handled.

++ means not quite so solid a clump.

++w means the clump breaks up partially while handling.

The other reactions are gradations of this reaction.

Be very careful not to read false clumping due to settling of cells or to rouleaux formation.

All trypsinated cell tubes will at first give the impression of a positive reaction, but a true positive reaction does not entirely disappear during handling. The weak reactions might be difficult to read, but if they stand for a while at room temperature, the clumping returns.

The titer is the reciprocal of the last tube in which a positive reaction occurs (except in the indirect blocking reaction, where the titer is the reciprocal of the last negative reaction). See example, Tables 328-330.

On all tubes where 2 tests are made, for example a Coombs test made after the saline agglutination test is negative or an indirect blocking test after the saline test is negative, the results are recorded by Unger as follows:

$\frac{-\mid}{-}$ means the first test was negative but the second test was also negative (top results in the box).

$\frac{+\mid}{-}$ means that the first test was negative but the second test (antiglobulin, etc.) was positive.

Note:

For the indirect blocking result the box opens to the right: $\frac{\mid +}{-}$

For the antiglobulin test it opens to the left: $\frac{+\mid}{-}$

Examples: See Tables 328-330.

In the indirect blocking titration, the last **negative** is the titer. If O Rh_1Rh_1 tubes are negative, remove saline and add anti-**Rh_o** tube serum. On other negative tubes make a Coombs test.

When negative, make a Coombs test after washing 3 times with saline.

In this example, Tables 328-330, the titers are as follows:

1. Indirect blocking: zero units.
2. Saline agglutinating: zero units.
3. Antiglobulin, 64 units against Rh_2rh.
4. Albumin-plasma method, 64 units against Rh_1Rh_1 and 128 units against Rh_2rh.
5. Trypsinated cell method, 128 units against Rh_1Rh_1 and also against Rh_2rh, and 1 unit against rh′.
6. Trypsinated-antiglobulin method, 256 units against Rh_1Rh_1 and Rh_2rh.

Table 328. *Rack 1: Saline-agglutinating, indirect blocking, and antiglobulin titer* (after Unger)

	1	*2*	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*512*	
O Rh_1Rh_1	$\frac{\mid +++s}{-}$	$\frac{\mid +++s}{-}$	$\frac{\mid +++s}{-}$	$\frac{\mid +++s}{-}$	$\frac{\mid +++s}{-}$	$\frac{\mid +++s}{-}$	$\frac{\mid +++s}{-}$	$\frac{\mid +++s}{-}$	$\frac{\mid +++s}{-}$	$\frac{\mid +++s}{-}$	No titer
O Rh_2rh	$\frac{+++s\mid}{-}$	$\frac{+++s\mid}{-}$	$\frac{++w\mid}{-}$	$\frac{++w\mid}{-}$	$\frac{+s\mid}{-}$	$\frac{+s\mid}{-}$	$\frac{+w\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	64
O rh′rh	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$						0
O rh″rh	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$						0
O rh	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$									0

Table 329. *Rack 2: Albumin-plasma titer* (after Unger)

	1	*2*	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*512*	
O Rh_1Rh_1	++s	++s	++s	++w	++w	+s	+	±	−	−	64
O Rh_2rh	++	++	++	++w	+s	+s	+w	+w	−	−	128
O rh′rh	−	−	−	−	−						0
O rh″rh	−	−	−	−	−						0
O rh	−	−									0

Table 330. *Rack 3: Trypsinated and trypsinated-antiglobulin titer* (after Unger)

	1	*2*	*4*	*8*	*16*	*32*	*64*	*128*	*256*	*512*	
O Rh_1Rh_1	++s	++s	++s	++	++	++w	+	+w	$\frac{+\mid}{-}$	$\frac{-\mid}{-}$	128-256
O Rh_2rh	+++	+++	+++	++s	++s	++	++	+s	$\frac{+\mid}{-}$	$\frac{-\mid}{-}$	128-256
O rh′rh	++w	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$						1
O rh″rh	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$						0
O rh	$\frac{-\mid}{-}$	$\frac{-\mid}{-}$									0

Note: If the serum gives a positive reaction against Rh-negative cells, an anti-**hr**′ or anti-**hr**″ antibody is present or some mistake has been made.

SIMPLIFIED TITRATION OF Rh ANTIBODIES IN SERUM FOR ROUTINE HOSPITAL USE

Although the foregoing method of titration and determination of specificity gives valuable information, routinely it is not necessary to conduct all 6 of the titrations; 3 should suffice: the saline agglutination titration method, the antihuman globulin titration method, and the trypsin-treated cell titration method.

When Rh-negative persons are sensitized by an Rh factor, the specificity of the antibody involved is usually anti-$\mathbf{Rh_0}$. Often anti-**rh′** is also present and far less frequently anti-**rh″**. For this reason it will suffice in routine practice to use untreated saline-suspended group O type Rh_zRh_0 cells and trypsinated saline-suspended group O type Rh_zRh_0 cells. Thus there will be only 2 rows of tubes for titrations and, in addition, the Rh-negative controls.

1. Make serial dilutions as directed under the method on p. 1529: 1:1, 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, and 1:512.
2. While making the dilutions place 1 drop of each dilution in its respective tube of 3 rows of tubes.
3. Add 1 drop of untreated saline-suspended group O type Rh_zRh_0 cells to each tube in the first row and 1 drop of group O type Rh_zRh_0 trypsinated cells to each tube of the second row.
4. In the third row, which should have 10 tubes (5 dilutions each for the negative controls), add 1 drop of the untreated saline-suspended group O rh cells to the first 5 tubes and 1 drop of trypsinated saline-suspended group O rh cells to the second 5 tubes (1:1, 1:2, 1:4, 1:8, 1:16). These are to detect Hr antibodies.
5. Incubate all tubes for 1 hr. at 37° C. in a water bath.
6. Read the bottoms of the tubes, and also read the reactions under a scanning lens.
7. Carry out antiglobulin tests on all negative reactions: Wash 3 times with saline. Remove all traces of saline after the last washing, using the technic outlined above.
8. Shake each tube and add 1 drop of Coombs serum (antihuman globulin) to each.
9. Centrifuge at 500-1000 r.p.m. for 2 min. and read.

The reciprocal of the highest dilution giving a positive reaction is the titer. See Tables 328-330 for recording the results.

PREPARATION OF ANTI-Rh SERUM FOR TESTING PURPOSES FROM NORMAL MALE DONORS (HISTORICAL)*

Because of the difficulty of securing an adequate amount of anti-Rh serum from isoimmunized women, Wiener and Gordon[158a] suggested the manufacture of this serum by injecting normal male professional blood donors. Their theory was based upon the fact that injections of relatively minute amounts of solutions A and B group substances into individuals lacking the corresponding agglutinogens stimulated a pronounced rise in the anti-**A** and anti-**B** agglutinin titers in such individuals.

Their method of immunization for the preparation of anti-Rh agglutinating serum was as follows: Group O, Rh-positive blood of a definite specificity is mixed with an equal volume of sterile citrate solution. The blood used in all experimental work belonged to type Rh_2Rh_2. Four ml. of this 50% blood suspension were injected intravenously into each of a number of donors. After an interval of 4 mo. (the maximum survival time for transfused red blood cells) a second intravenous injection was given, and 10 days later blood was withdrawn for testing. Donors with a satisfactory titer of anti-Rh agglutinins were bled.

The original work comprised the injection of 9 donors. Of these, 4 showed no evidence of antibody formation, whereas 5 had anti-Rh agglutinins in their sera. In 2 donors the titers of anti-Rh agglutinins were very high, 200 and 600 units, respectively. By the method of exchange transfusion with type rh donors, 1500 ml. blood were removed from each man, and a satisfactory yield of testing serum was obtained.

For more recent statistics see Wiener.[159]

TEST FOR Hr ANTIBODIES IN SERUM

The test for Hr antibodies in serum employs ficinated hr′ and hr″ cells belonging to group O. It is not made on serum when the individual has an rh-negative gene, but patients or donors of types Rh_1Rh_1, Rh_2Rh_2, and others without the *r* gene may be tested. The rapid ficinated technic of Wiener is used.

Before making the test, determine first that the serum to be tested does not contain anti-$\mathbf{Rh_0}$, anti-**rh′**, or anti-**rh″** agglutinins (refer to pp. 1522ff).

Procedure:

1. Prepare suspensions of group O hr′ (Rh_2Rh_2), group O hr″ (Rh_1Rh_1), and group O rh (**hr**′ and **hr**″ positive) cells in saline, wash twice

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*It is best to purchase these sera from United States Government licensed manufacturers.

Table 331. *Results possible when testing unknown serum for Hr sensitization*

	hr″ (Rh_1Rh_1) cells	*hr′ (Rh_2Rh_2) cells*	*rh cells*	*Conclusions*
Unknown serum	−	+	+	hr′ sensitization
Unknown serum	+	−	+	hr″ sensitization
Unknown serum	−	−	−	No hr′-hr″ sensitization
Unknown serum	+	+	+	hr′ and hr″ sensitization

with saline, and discard the supernatant fluid after the last washing. Resuspend to 3-5% concentration in saline.

2. Place 2 drops of saline-suspended group O hr′ cells (Rh_2Rh_2) in a small Kahn tube properly labeled.
3. Place 2 drops of saline-suspended group O hr″ (Rh_1Rh_1) cells in a small Kahn tube properly labeled.
4. Place 2 drops of the saline-suspended group O rh (**hr′** and **hr″** positive) cells in a small Kahn tube properly labeled.
5. Add 1 drop of diluted ficin, 1:5 (p. 1463), to each tube and mix.
6. Place the 3 tubes in a water bath at 37° C. for 15 min.
7. Wash the contents of each tube once with saline, and after the last centrifugation remove and discard the supernatant fluid.
8. Add 1 large drop of the unknown serum to each tube.
9. Mix and place in a water bath at 37° C. for 30 min.
10. Read for agglutination or lack of agglutination, first gently dislodging the sediment. Read microscopically and under a scanning lens of a microscope or under a hand lens.

If there is no agglutination, wash the contents of each tube 3 times with saline, and after the last centrifugation remove and discard the supernatant saline. All traces of saline must be removed.

Add 1 drop of antiglobulin serum (Coombs) to each tube, shake, and centrifuge at 1000 r.p.m. for 1 min. Observe for clumping or lack of clumping after gently dislodging the sediment.

Results—to determine specificity of Hr antibody present: Assume that a patient belongs to phenotype Rh_1Rh_1. If there is no evidence of sensitization to **Rh**$_0$, **rh′**, or **rh″** as proved by previous tests, the following conclusions may be drawn from this test.

Rh_1Rh_1 cells have the **hr″** antigen; therefore, if these cells are not agglutinated by the unknown serum, the unknown serum lacks **hr″** antibody (as well as anti-**Rh**$_0$ and anti-**rh′**), and the unknown is not sensitized to **hr″**.

Rh_2Rh_2 cells have the **hr′** antigen; therefore, if these cells are not agglutinated by the unknown serum, the unknown serum lacks **hr′** antibody (as well as anti-**Rh**$_0$ and anti-**rh″**), and the unknown is not sensitized to **hr′**.

If the unknown serum reacts negatively with rh cells, there is no **hr′** or **hr″** sensitization and therefore no anti-**hr′** or anti-**hr″** agglutinins in the unknown serum.

If the unknown serum reacts positively against Rh_2Rh_2 cells and no **rh″** or **Rh**$_0$ sensitization has been shown by prior tests, then this serum has anti-**hr′** agglutinins.

If the unknown serum reacts positively against Rh_1Rh_1 cells and no **rh′** or **Rh**$_0$ sensitization has been shown by prior tests, then this serum has anti-**hr″** agglutinins.

If the unknown serum reacts positively against type rh cells, there is either **hr′** or **hr″** sensitization, or both. If there was no reaction against Rh_1Rh_1 (**hr″** positive) cells, but there was a positive reaction against Rh_2Rh_2 cells (**hr′** positive), and the positive reaction with type rh cells shows the presence of an **hr′** or **hr″** antibody, by subtracting **hr″** from **hr′** + **hr″**, this leaves **hr′** sensitization as proved by the test using Rh_2Rh_2 cells. The group O rh cells act as a control over the other 2 reactions.

This test shows specific sensitization of an Rh-positive patient to an Hr factor when no **rh′** or **rh″** sensitization has been shown to exist when testing the Rh-positive serum against rh′ and rh″ cells.

An individual with phenotype Rh_1Rh_1 gives the following reactions of cells against specific antisera:

Anti-**rh′**	Anti-**rh″**	Anti-**Rh**$_0$	Anti-**hr′**	Anti-**hr″**
+	−	+	−	+

and such an individual cannot be sensitized against **hr″** but can be sensitized to **hr′**. For possible results consult Table 331.

Chapter 77

Erythroblastosis fetalis

ADDINE G. ERSKINE

The discovery of the Rh factor supplied the key to 2 medical mysteries[160]*—the cause of hemolytic transfusion reactions in patients receiving blood of their own A-B-O group and the pathogenesis of erythroblastosis fetalis. The demonstration by Wiener of the existence of 2 forms of antibodies, the agglutinating or "bivalent" antibody, which reacts in saline media, and the blocking or "univalent" antibody, which reacts in high protein media, that is, the reaction takes place in saline and is detectable by the antiglobulin technic, led to new tests for the detection of isoimmunization. Since the original work of Landsteiner and Wiener in 1937, many different varieties of Rh factors have been discovered, as well as certain contrasting factors known as Hr factors and the factors of other blood group systems. It is now possible by testing the blood of the expectant mother and her husband and by carrying out antibody tests on the expectant mother's serum periodically during the pregnancy to predict not only what the newborn baby's blood type may be but also whether or not the baby is likely to have hemolytic disease.

Erythroblastosis fetalis is characterized by abnormal destruction and regeneration of erythrocytes. It is fair to assume that intravascular hemolysis or clumping, or both, initiates all the other pathologic changes in this condition. Much of the pathology depends upon an excessive destruction of erythrocytes and upon an attempt on the part of the body to compensate for this loss. Anemia, increased bilirubin in the blood and in various fixed tissues of the body, abnormal erythropoiesis in many organs, including the spleen and liver, and an increase in immature forms of erythrocytes in the circulating blood are among the signs most commonly observed.* The extreme edema present in some of these cases has not as yet been satisfactorily explained. Some have explained it on the basis of renal failure, injury of the general capillary bed, and a decrease in plasma proteins occurring in conjunction with cellular hemolysis.

The clinical and laboratory manifestations include jaundice, anemia, erythroblastemia, edema, hepatosplenomegaly, and purpura, varying widely in degree. The infant may have none, few, or all of these signs. The most severely affected infants are either stillborn or die shortly after birth. In those untreated babies who survive the immediate postnatal period there is almost invariably a steady rise in the concentration of the blood bilirubin, which is believed to occur because the liver of the newborn infant is unable to convert indirect into direct bilirubin.

According to Wiener and Wexler,[161] the clinical course of these babies can be predicted in only a general way. Those with severe anemia, petechiasis, high cord blood icterus index, edema, and hepatosplenomegaly generally die within a few hours or days with pulmonary hemorrhage after becoming extremely jaundiced. Postmortem examination in such cases will almost always show bilirubin staining of the basal nuclei of the brain, **kernicterus.** Other infants who appear less severely affected at birth, with a more moder-

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*For a description of the blood picture in erythroblastosis fetalis, see p. 1295.

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ate anemia, 10-14 gm.% hemoglobin, but who have an elevated cord blood icterus index, more than 16 units, will develop intense jaundice and similarly die within 3-5 days with nuclear jaundice. In this group some babies may survive, but because of brain damage they later exhibit retarded development.

Of the infants who appear to be more mildly affected (normal cord blood, blood hemoglobin, cord blood icterus index less than 16 units), so that life is not in jeopardy, nuclear staining may nevertheless still occur and some degree of cerebral damage may become manifest either during the neonatal period or later in life. All such affected infants should therefore be treated with exchange transfusion which to date is the only treatment that has been proved to save the lives of these babies and prevent neurologic sequelae.

There is a group of infants whose general condition remains good and whose jaundice never becomes intense enough to be a cause of concern. Such babies should be treated expectantly. It must be stressed, however, that in case of doubt it is better to perform an exchange transfusion than to leave untreated any baby who might sustain brain damage.

Effective methods have been devised for treating the affected erythroblastotic baby,[162] particularly the use of exchange transfusion. If the baby at birth is not already moribund or irreversibly damaged, it is generally possible to save him with exchange transfusion. In babies who survive it is possible almost invariably to prevent the occurrence of any neurologic sequelae. The success of treatment depends on the promptness and thoroughness with which it is carried out. If the prenatal tests indicate the likelihood of an erythroblastotic baby, a trained immunohematologist should be consulted during the pregnancy, to be available when the baby is born. Prophylactic treatment of the sensitized Rh-negative mother with so-called "Rh-hapten," diethylenesulfonate, progesterone, exchange transfusions of the mother, cortisone, and "desensitizing" injections of Rh-positive blood have been unsuccessful. The only treatment that has been successful to date is exchange transfusion of the affected baby. In this transfusion the baby's coated cells and some of his plasma containing univalent antibodies derived from the mother are removed while being replaced with erythrocytes which cannot be coated by the offending antibodies.

Although the great majority of cases of erythroblastosis fetalis are the result of $\mathbf{Rh_0}$ sensitization, not infrequently Rh-positive as well as Rh-negative mothers may have erythroblastotic babies because of A-B-O sensitization. In addition, sensitization has been found to be due to any of the numerous Rh-Hr factors, particularly **hr′**. Sensitization to blood factors of other blood systems, such as **Kell** (**K-k**), have been reported, and in rare instances there has been sensitization to factors **P**, **U**, $\mathbf{hr^S}$, **M**, and others. It is not always practicable for the laboratory to test specifically for sensitization to each of the factors, and for that reason sensitization tests of the mother's serum should be made using cell panels as well as cells containing all the Rh-Hr factors. Most of the cases, as stated above, result from $\mathbf{Rh_0}$ sensitization, and the other cases can be diagnosed after the baby is born by observing all newborn babies closely for evidence of early jaundice or anemia. When jaundice and anemia appear during the first or second day of life, immunohematologic tests are necessary in order to save the baby's life.

It is common practice today in many hospitals to perform a direct Coombs (antiglobulin) test on the cord or infant's cells as soon as the baby is born, and if the test is positive and if the cord bilirubin is significantly elevated, an exchange transfusion is performed. Often the physician will wait 8-12 hr. or longer to see what signs or symptoms develop, according to Wiener.[163] If the baby shows no **clinical** signs, for example, no jaundice and no anemia, an exchange transfusion is not performed.

The principal factor in determining the possible severity of the disease is the mother's antibody titer, because it in turn determines the degree of coating of the fetal cells (Table 332). Simple coating of the fetal red cells need not necessarily interfere with their function; not only must they be coated but conglutinin must also be present before clumping takes place. Although complement and complement-like substances are not fully developed before birth, if the mother's antibody titer has been high for sufficient time, the child may be stillborn or, if born alive, may manifest symptoms of severe erythroblastosis fetalis. Often the baby appears normal at birth, but early in the neonatal period symptoms appear.

There is no direct relationship between the depth of jaundice and the degree of anemia. The jaundice is hepatogenous as well as hemolytic in origin. In addition, although brain damage occurs more often in severely jaun-

Table 332. *Results of Rh antibody titrations carried out on the expectant mother**

Date of test	*Agglutination method*	*Albumin-plasma conglutination method*	*Antiglobulin method*	*Ficinated cell method*
1/11/58	3	30	144	192
1/18/58	2½	0	80	320
1/27/58	16	280	146	400
2/ 3/58	5	110	176	196
Average of antenatal tests	6½	140	136	277
3/1/58 (postpartum)	0	68	160	128

*From Wiener, A. S.: Advances in blood grouping, New York, 1961, Grune & Stratton, Inc., p. 234.

diced babies, some severely jaundiced babies may recover completely and some with lighter jaundice may die, with brain damage. Wiener[164] believes that the tissue damage is due primarily to vascular injury resulting from intravascular conglutination of the baby's coated cells. Damage to the liver results in jaundice. Blockage of circulation in the brain produces nuclear damage and allows bilirubin to cross the blood-brain barrier and stain the dead and dying ganglion cells; therefore, if the coated cells are removed before intravascular clumping occurs and are replaced by cells that cannot be coated by the free antibody, tissue damage may be prevented. Tissue damage which may be present at birth appears to be largely reversible. Kernicterus is rarely if ever found in newborn or stillborn infants. This is to some degree probably due to the fact that bilirubin formed in utero passes into the maternal circulation and is largely excreted. The rapidly developing jaundice and organic injury are probably due to increase in the concentration of complement or conglutinin in the infant's circulation during the early neonatal period. This results in intravascular clumping and hemolysis.

HYPERBILIRUBINEMIA AS AN INDICATION FOR SECOND EXCHANGE TRANSFUSION

In a case described by Wiener,[165, 166] differential agglutination tests carried out after the exchange transfusion showed that there had been virtually a complete replacement of the baby's Rh-positive coated red blood cells by type rh cells. The principal purpose of the exchange transfusion had therefore been accomplished—to remove the baby's coated cells and to replace them with other red cells incapable of reacting with the Rh antibodies passively acquired by the baby by placental transfer from the mother. However, it is not sufficient merely to remove the baby's coated red cells, which cause the disease, because the disease process has a certain momentum and it may require a few days for the damage to be reversed. In the meantime, because of the poor liver function in the newborn infant, bilirubin may accumulate in the baby's body, and if the bilirubin concentration reaches a high enough level, irreversible brain damage may result. It is therefore necessary at times to carry out a second exchange transfusion to counteract the hyperbilirubinemia until such time as the baby's liver can cope with the bilirubin.

Wiener follows the bilirubin level by icterus index determinations rather than by the van den Bergh tests, using acetone precipitation of serum which also precipitates any contaminating hemoglobin. After centrifugation, the supernatant fluid is diluted with water and matched against a series of potassium dichromate standards. See p. 101, Dingee method. Icterus index values are converted into bilirubin concentration by dividing by 6. Brain damage rarely results before the icterus index exceeds 150-200 units, corresponding to a bilirubin level of about 25-30 mg.%. A figure of 120 icterus index units or about 20 mg.% of bilirubin would therefore be set as an indication for repeating an exchange transfusion, which leaves a safe margin of error.

In the case cited by Wiener the icterus index of the cord serum was 30 units as compared with a maximum normal value of approximately 15 units. The first sample of blood taken at the exchange transfusion 1 hr. later had an icterus index of 36 units, whereas a sample obtained at the end of the transfusion had an icterus index of 18 units. There was a drop of only about 50% in the icterus index value in contrast to the approximate 98% replacement of red cells in the exchange transfusion (2 units of blood were used). There were 2 reasons for this: (1) bilirubin is present not only in the blood plasma but also in the extravascular tissue spaces from which it diffuses into the circulation during the exchange transfusion and (2) the concentration of bilirubin is not static but dynamic. The bilirubin concentration, not only in erythroblastotic babies but in normal babies as well, tends to rise during the first 2-4 days of life.[167] Twenty hours after the exchange transfusion the icterus index in this case had risen to 60 units; at 44 hr. it was 100 units, and at 68 hr. it was 120 units. Preparations were therefore made for a second exchange transfusion. Two units of type rh blood, partially concentrated, were obtained. At 74 hr. and prior to the second exchange transfusion another sample of blood showed an icterus index of 144 units.

A second exchange transfusion was carried out by the radial artery–saphenous vein technic, using the blood vessels on the opposite side. About 800 ml. blood were injected over a period of 1½ hr. through the saphenous vein at the ankle while an equal volume was withdrawn from the radial artery at the wrist. The baby withstood the procedure well and was returned to the nursery in good condition though still deeply jaundiced.

A sample of blood withdrawn at the midway mark had an icterus index of 90 units, and the last sample taken at the exchange was only 72 units. The following morning the icterus index was only 78 units, and the baby appeared less jaundiced. The jaundice faded rapidly thereafter; after 48 hr. there was no discernible jaundice and the baby was sent home, to be followed at the office.

Some workers, in treating severely affected erythroblastotic infants, have given as many as 7 exchange transfusions on successive days, using only 1 unit of blood for each transfusion. However, there is only a slight drop in bilirubin concentration with a rebound often to a considerably higher level than before the exchange transfusion. Not every infant requires more than 1 transfusion.

AMOUNT OF ANTIBODY AND BILIRUBIN REMOVED BY EXCHANGE TRANSFUSION

The antibody level in a baby depends on the amount acquired from the mother by passive placental transfer since the baby does not produce any antibody. Such antibodies have been found[166] to have a half-life in the baby's circulation of approximately 30-35 days. In erythroblastotic babies the half-life may be less since newly formed Rh-positive cells can absorb the free Rh antibodies.

Wiener calls attention to the **rebound phenomenon**, that is, the rise in antibody titer back almost to the original level in the days following an exchange transfusion. Considering that the half-life of the Rh antibody is about 30 days, a titer of 12 units obtained when the baby is 1 mo. old represents a titer of at least 24 units at birth, especially since the increase in the baby's size would tend to dilute the antibody and lower the titer further. In one erythroblastotic baby the initial titer value was 44 units, and 2 exchange transfusions totaling about 8 times the baby's blood volume were given, which removed only approximately half the Rh antibodies. This gives some indication of the large size of the extravascular space containing free antibodies and presumably also bilirubin. The routine use of only 1 unit of blood for an exchange transfusion may not produce the desired effect, and in severely affected babies at least 2 units should be used.

EXCHANGE TRANSFUSION

As Wiener first showed, the pathognomonic serologic finding of Rh hemolytic disease is the positive direct antiglobulin reaction of the baby's red cells (direct Coombs test). In A-B-O hemolytic disease the direct antiglobulin test is negative or it may be only weakly positive. Maternal antibodies passing through the placental barrier become attached to fetal red cells, and these coated red cells are believed to initiate the disease process. Theoretically, therefore, removal of the baby's coated red cells and replacement by red cells incapable of combining with maternal antibody would be expected to arrest the disease process.

In 1944 Wiener et al. adopted a method of treating erythroblastosis fetalis by simultaneously withdrawing and injecting blood, a procedure that they designated exchange transfusion.[162, 168] Since in exchange transfusions blood is withdrawn at the same time that compatible blood is given, from the onset some of the transfused cells are removed. It is for this reason that an exchange transfusion involving a quantity of blood equal only to the baby's blood volume will be insufficient to accomplish a satisfactory exchange. A pint of blood (approximately 2 blood volumes) will accomplish an 85% replacement of red blood cells. Wiener prefers, except in the mildest cases, to use 2 pints of blood. In a baby of average size, with an approximate blood volume of 250 ml., this quantity will accomplish about a 98% replacement. Unger, on the other hand, prefers to remove, if possible, 80 ml. of the infant's blood before starting the exchange. He then exchanges only 1 unit of blood. He very rarely finds it necessary to do a second exchange.

A blood bilirubin concentration of 20 mg.% at any time during the first 72 hr. of life, as previously explained, may be regarded as an indication for repeat exchange transfusion, especially if the rate of rise indicates that even higher levels will probably be reached. An attempt is made to delay the second exchange transfusion as long as it is safe to do so and then, depending on the severity of the case, to use 2 or even 3 units of blood. When this method is followed, there has seldom been any need for a third exchange transfusion. Indications for a second exchange transfusion are usually evident by the time the baby is 24-48 hr. old. Serial determinations of blood bilirubin must be made as a guide for treatment, as explained on pp. 1536 and 1537.

Donor's blood: Obtain the freshest possible blood for the transfusion. It should never be more than 3 days old. In Rh hemolytic disease use Rh-negative blood, which must be compatible with either the baby's serum or with the maternal serum. Low-titered group O Rh-negative blood may also be used in all cases. Unger disregards the titer of anti-**A** and anti-**B** in the group O blood used because he first removes almost all of the supernatant plasma. It is best, however, to avoid depletion of the supply of group O Rh-negative donor blood, so that Rh-negative blood of the **mother**'s A-B-O group is used, since this is always compatible with the infant's serum, even when the baby is group O and the mother is group A or group B. Such blood can be prepared before the baby is born.

In cases of A-B-O incompatibility, low-titered group O blood is used. In the very rare instances in which the antigen responsible for the incompatibility is not immediately identified and the infant's clinical condition is desperate, the mother may be bled and her blood cells used to treat the baby.

Before the citrated blood is used for the exchange transfusion, it is concentrated to 450 ml. by withdrawing 100-150 ml. plasma after the blood has been allowed to sediment while standing in a refrigerator. Partially concentrated blood prepared in this manner has a hematocrit value of about 50%.

Procedure—Wiener-Wexler:

1. Immobilize the infant on a circumcision board by the use of flannel wrappings, leaving one arm and one leg free.
2. Strap down the foot with adhesive tape and expose the saphenous vein at the ankle through a transverse incision made anterior and superior to the medial malleolus.
3. Cannulate the vein with a 20-gauge spinal trocar and tie in place.
4. Inject 200 units heparin, diluted in 2 ml. saline, through the needle and attach a 3-way stopcock. This in turn bears the tubing from the blood reservoir as well as a 10 ml. Luer-Lok syringe for injection of the blood and the medication.
5. Before any injection of blood is made, isolate the radial artery through a transverse incision on the radial side of the wrist of the free arm just proximal to the head of the radius.
6. Expose the artery by gentle, blunt dissection. It can be readily recognized as a moderate-sized, plump vessel, the wall of which is a little thicker than that of the saphenous vein at the ankle.
7. Carefully clean the artery, lift up on a closed mosquito clamp, and open by making an angled incision through the flattened wall. The artery is not cannulated since this would impede the flow of blood.
8. Collect the blood in 30 ml. medicine glasses, which in turn are emptied into a 1 L. graduated container for final measurement.
9. To increase the efficiency of the exchange transfusion first bleed the infant of about 60 ml. before administering the blood.
10. Commence the injection of blood through the saphenous vein at a rate that keeps pace with the arterial flow and that makes up for the deficit produced by the initial bleeding by the time the 90 ml. mark is reached. Equal quantities of blood are administered and withdrawn.
11. During the procedure give a repeat dose of heparin at the 200 ml. mark and, if 2 units are used, at the beginning of the second unit.
12. Beginning with the 50 ml. mark, administer 1.5 ml. of 10% calcium gluconate solution for each 100 ml. blood given. Inject this directly into the venous trocar and immediately flush through with 10 ml. blood.
13. At the termination of the procedure close the incisions with fine silk sutures, which are removed on the fourth postoperative day. Pressure bandages control bleeding. Neither the artery nor the vein is ligated, and it may be assumed that they recannulate.

Postoperatively, place the infant into an Isolette for warmth. Oxygen may be given if needed. Prophylactic penicillin, 100,000 units every 8 hr. for 2 days, is the only routine medication used. Feedings are standard, and the more robust infants may be breast fed.

If the procedure is repeated, the opposite arm and leg are generally used. It is possible to recannulate the vein for the second transfusion, but the artery will not bleed freely again after it has been used for the first transfusion.

In Wiener and Wexler's experience no mortality that could be ascribed to the procedure itself has occurred with this technic. The only limitation to the speed with which the operation is carried out is the rate at which the blood flows from the arterial end. The shortest time for 1 unit of blood has been 25 min. and the longest 2 hr., in the experience of Wiener and Wexler. Since the inflow and outflow are always balanced, blood volume changes do not occur, and the babies appear to be quite comfortable throughout the procedure.

Wiener et al. have cautioned against the indiscriminate use of the umbilical catheter method of exchange transfusion, stating that it is a blind procedure and has been said to have caused damage to important intra-abdominal vessels with resultant thrombosis and embolism. Intra-abdominal hemorrhage, peritonitis, and splenic rupture have also been reported following its use. In view of these dangers, the assertion that the method is easier to perform is not sufficient justification for its use; moreover, this assertion is not in the strictest sense true, since not infrequently the umbilical vessels cannot be catheterized and operators have at times had to resort to incisions in the abdominal wall and groin in order to catheterize large vessels in those regions.

Booster transfusion: Wiener[160] has pointed out the need for a booster transfusion in cases in which the baby's plasma contains a high titer of free Rh antibodies. When there are free Rh antibodies in the baby's plasma, the baby's newly formed Rh-positive red cells may become partly coated, and when the baby is 3-4 wk. old, his blood may give a weakly positive direct antiglobulin (Coombs) reaction. This is generally harmless. Eventually the red cells will all be Rh positive and the direct antiglobulin test will become negative. However, when the Rh antibody titer is high, the baby's Rh-positive cells may be destroyed as rapidly as they are released from the bone marrow. Such babies may require a booster transfusion.

The most convenient donor to use for the booster transfusion is the mother, since her cells are always compatible. It is necessary, however, to remove the supernatant plasma containing Rh antibodies and to wash her cells with sterile saline solution. The father's blood cannot be used since he is Rh positive and his infused cells could become coated with the free antibodies in the baby's blood.

SENSITIZATION TO THE A-B-O FACTORS*

Halbrecht,[170] Polayes and McNally,[171] and Wiener et al.[172] called attention to cases of A-B-O sensitization in which the Rh factor had no part whatsoever. Mild cases of erythroblastosis due to A-B-O incompatibility appear to be quite common but are usually overlooked. Although most infants recover spontaneously, in some cases the manifestations are severe enough to warrant treatment.

It was noticed first by Rosenfield[173] that erythroblastosis due to A-B-O incompatibility is almost always confined to babies of group O mothers. This can readily be seen in earlier reports on series of cases by Wiener, Sonn, and Hurst and by Wiener, Wexler, and Hurst. Wiener and Unger showed that group O babies of group O mothers acquire by placental transfer about 16 times as much isoantibody as do babies having mothers of group A or group B. They concluded that this was adequate to account for the excess of group O mothers with babies having A-B-O hemolytic disease.

As explained elsewhere in this section (p. 1472), agglutinogens A and B are both characterized by factor **C**, so that there is no anti-**C** present in the sera of groups A, B, and AB. Group O erythrocytes are the only ones that do not contain the **C** factor, and therefore group O serum is the only variety that can have antibodies specific for factor **C**. These antibodies, Unger and Wiener showed,[174, 175] are usually of the univalent variety and therefore readily traverse the placental barrier. Thus, if the mother's blood is group O and that of the fetus is group A, the fetal erythrocytes have the **C** factor, whereas the maternal erythrocytes lack it, and anti-**C** may be formed in excess by the mother if the group A blood cells of the fetus enter the maternal circulation. These antibodies in turn traverse the placenta and enter the fetal circulation. Since these **C** antibodies cross react with both A and B agglutinogens, if these agglutinins are present in the baby's plasma in sufficient amount, they could produce hemolytic disease in the infant.

Wiener[176] concluded that (1) the A-B-O blood properties appear to be the most com-

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*Refer to p. 1472 for discussion of factor **C** of the A-B-O blood groups.

mon cause of erythroblastosis in infants with Rh-positive mothers; (2) the natural anti-**A** and anti-**B** agglutinins in maternal sera, as a rule, have little or no harmful effect on fetuses belonging to groups A, B, and AB, because they usually are of low titer and avidity and because the natural isoagglutinins presumably are larger molecular aggregates which do not traverse the placenta easily; (3) individuals may become isosensitized against the A and B properties by the inadvertent transfusion of blood of an incompatible group, from pregnancy with a fetus of an incompatible group, or by injections of materials containing soluble group substances such as pooled human serum, therapeutic horse serum, etc.; (4) immune isoantibodies for A and B in the maternal serum (anti-**C**) are more dangerous for the fetus than the natural isoagglutinins because they are more likely to be of high titer and, in addition, pass across the placenta more readily; (5) cases of congenital hemolytic disease due to A-B-O sensitization are usually milder than cases due to Rh sensitization, and patients frequently recover without treatment, but severe or even fatal cases, with liver damage and kernicterus, also occur, and in these cases the A-B-O factors are the cause; (6) when Rh-negative individuals are exposed to Rh-positive blood of an incompatible blood group, the principle of competition of antigens may operate, so that the agglutinogens A or B may suppress the antigenicity of the Rh factor.

In the case of pregnancy in a union between an Rh-positive husband and an Rh-negative wife, one may expect the first-born baby to be free from symptoms. Physicians should be ready to proceed with the proper treatment of children of an Rh-positive father and an Rh-negative mother by exchange transfusion with Rh-negative blood.

A-B-O hemolytic disease, in contrast to that caused by the Rh factor, frequently affects the first-born infant.[162, 177-179]

Chapter 78

Other blood group systems

ADDINE G. ERSKINE

LEWIS BLOOD GROUPS AND TESTS FOR A-B-O SECRETORS—FACTORS Le^a AND Le^b

In 1946 Mourant[180] found in the serum of a blood donor an antibody that agglutinated the blood cells of 24 out of 96 random individuals belonging to group O. This antibody possessed properties of type-specific cold agglutinins in that the reaction with it was stronger at low temperatures than at body temperature. The blood factor detected by this serum was named **Lewis** (**Le**) after one of the donors whose serum contained the antibody. An identical antibody was found in 1948 in Denmark by Andresen,[181] who shortly thereafter found a second type of serum which gave almost antithetical reactions. The factor identified by Mourant's antiserum is called **Le**^{a}, whereas that which appears to be allelic is called **Le**^{b}. Many examples of the **Le**^{b} antibody have now been found, and most of these give good positive reactions only if the cells being tested are group O or group A_2.[182]

In 1948 Grubb[183] reported that practically all persons who have the **Le**^{a} factor on their red cells are nonsecretors of the A-B-O (H) blood group substances, whereas those lacking **Le**^{a} are almost always secretors. The words "secretors" and "nonsecretors" are used to refer to individuals who secrete the antigens **A**, **B**, and **O** (H). The Lewis substance is a mucopolysaccharide chemically indistinguishable by present methods of identification from A-B-O substances.[184, 185]

The A-B-O factors are not restricted to the red cells but are also present in the cells of tissues and organs with the exception of nerve tissue, epithelium, skin appendages, bone, and cartilage. The blood group substances present in red blood cells and tissue cells can be extracted with alcohol and other lipid solvents, but only in small amounts. Since the blood group substances also occur in water-soluble form, all persons can be divided into 2 groups, those who secrete the substance, called secretors, and those who do not, called nonsecretors. The blood group substances can be extracted with aqueous solutions from tissues and organs, especially salivary glands and gastric mucosa, of secretors, and these substances are present in high concentration in saliva, gastric juice, and semen. Water-soluble group substances are not demonstrable in the body fluids or secretions of the nonsecretors. It has been shown that the saliva of **Le**^{a}-positive individuals is capable of neutralizing anti-**Le**^{a} serum.

About 75-80% of Caucasoids are secretors. Approximately 80% of infants less than 3 mo. of age have the **Le**(**a**+) factor on their red cells (nonsecretors), but by the end of the first year of life this frequency drops to 20%, the adult level. Unger noted that all cord blood specimens which he examined were **Le**(**a**–). It has been found that the more the **Le**(**a**+) cells are washed with saline the less they are agglutinable by anti-**Le**^{a} serum, although Race and Sanger were not able to remove **Le**(**a**+) by washing. This observation might necessarily depend on the antiserum used in testing the cells.

According to Andresen,[186] 4 phenotypes are present in the Lewis system, which can be determined by 3 agglutinins—anti-**Le**^{a}, anti-**Le**^{b}, and one that has received the temporary name of anti-**X**:

Le(a+b–) are always nonsecretors.

Le(a–b+) are always secretors.

Le(a–b–) are always secretors when they give a positive reaction with anti-**X**. Such individuals are found only in group A_1.

Le(a–b–) which give negative reactions with anti-**X** are either secretors or nonsecretors.

In the case of the Lewis system, the phenotypes apply only to adults. Other rules apply to infants below the age of about 1 yr. **Le**^{b} is typically dominant in adults, and this property is faintly developed in infants. **Le(a+b+)** is very common among small children but has never been demonstrated in adults. Perhaps the use of more potent anti-**Le**^{a} serum in the future may reveal this type in adults also.

Transmission of **Le**^{a} parallels that of the secretor/nonsecretor trait. Two Le(a+) parents, both nonsecretors of the A-B-O substance, have all Le(a+) children, also nonsecretors. If both parents are Le(a–), the children may be Le(a+) as well as Le(a–); the Lewis factor behaves like a mendelian recessive.

The Lewis substance appears to be produced primarily in the tissues and body fluids and secondarily adsorbed onto the red cells.

When the red cells are tested with both anti-**Le**^{a} and anti-**Le**^{b} sera, conflicting results are obtained, perhaps because anti-**Le**^{b} sera give many more positive reactions with cells of groups O and A_2 than with cells of groups A_1 and B. When tests are confined to blood cells of groups O and A_2, **Le**^{a} and **Le**^{b} are almost, but not completely, antithetical. The presence of **Le**^{a} on the red cells is demonstrable only in homozygous individuals, genotype Le^aLe^a. This may represent a gene dosage effect (Wiener) since all available anti-**Le**^{a} sera to date have been of very low titer and avidity.

The **Lewis** factor is important in genetic and family studies and in medicolegal practice to determine secretors and nonsecretors of the A-B-O substance. The significance of the **Lewis** factor in blood transfusions is that natural or immune antibodies for all the blood factors may produce unexpected agglutinations, making it difficult to obtain compatible blood for transfusions. The primary purpose of testing for the **Le** factor, however, is to determine secretors and nonsecretors, important in medicolegal blood grouping serology, especially when blood group substances can be demonstrated in saliva on cigarette stubs or postage stamps, and in seminal stains in rape cases. The concentration of the blood group substances in such materials is relatively high, and the results of the tests are therefore usually clear-cut. The principle of these tests is to determine the capacity of saline extracts to inhibit specifically the reactions of anti-**A** and anti-**B** sera. This method has also been applied successfully to blood grouping of corpses when there was no blood available with which to carry out direct agglutination tests. In such cases saline extracts of muscle tissue are prepared and used for the test. Errors in **Lewis** testing are avoided by including suitable controls. Tests on tissues and secretions are restricted to the A-B-O groups.

Testing for Le^a

In testing for the **Le**^{a} blood factor, certain antisera require that the unknown cells be enzyme-treated, whereas other sera do not. If the anti-**Le**^{a} serum requires enzyme-treated cells, it will react with the cells both at 4° C. and at room temperature, but those sera that do not require enzyme-treated cells react only at refrigerator temperature.

1. Prepare cell suspensions according to the directions on p. 1463, wash twice, and resuspend to a 3-5% concentration. If the antiserum requires enzyme-treated cells, prepare ficinated cells according to the short method on p. 1463.
2. Place 1 drop of cell suspension of unknown blood in a small Kahn tube.
3. As a control place 1 drop of Le(a+) cells in a small Kahn tube and 1 drop of Le(a–) cells in another tube, both properly labeled.
4. Add 1 drop of anti-**Le**^{a} serum to each of the 3 tubes and mix thoroughly.
5. Place the tubes in a refrigerator at 4° C. for 1 hr. At the end of this period, read the results as positive if clumping has occurred or as negative if no clumping has taken place. Read the reactions in the control tubes and use these as comparisons in judging the results. Do not make antiglobulin (Coombs) tests when testing for the **Le**^{a} factor.

Considerable difficulty has been encountered in carrying out tests for the **Lewis** factor. Another test has been devised for determination of group substance O secretors and nonsecretors. The method makes use of a reagent prepared from a seed, *Ulex europaeus*. The reagent is called "anti-**H** Lectin" and is available commercially.* It is not specific for blood group O because the *Ulex* seed extract, like anti-O sera in general, tends to react most strongly not only with red cells from individuals of group O but also of subgroup A_2. Because of this characteristic, the serum is called anti-**H** and not anti-**O**.

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*Certified Blood Donor Service, Inc., Jamaica, L. I., N. Y.

As previously stated, the A-B-O factors are not restricted to red blood cells, but in approximately 80% of adults they are also present in water-soluble form in body fluids and in such secretions as saliva, gastric juice, nasal secretion, semen, and amniotic fluid. Those who have these substances in their secretions are secretors and those who lack the substances are nonsecretors. Schiff and Sasaki have shown that the secretor trait is inherited by a pair of allelic genes, independent of the A-B-O groups. Individuals can be classified as secretors or nonsecretors by testing the saliva. The method of testing saliva is by the inhibitory method.

Inhibitory test of saliva, semen, and other body fluids for detection of A-B-O secretors

A-B-O secretors

In this test the blood group-specific substance of saliva is used to neutralize the anti-**A** or anti-**B** agglutinin of serum. If the anti-**A** agglutinin is inhibited, addition of group A cells will result in absence of agglutination. Similarly, if anti-**B** is inhibited, the antiserum will no longer agglutinate group B cells.

The agglutinin inhibitory test may be used for determining the blood group of semen, saliva, or other body fluids but may not be used for blood.

Since saliva contains enzymes that can destroy the A-B-O blood group substances, as soon as the specimen is collected it must be placed in a boiling water bath for 10 min. or brought to a boil over a Bunsen flame. Remove the coagulated mucus by centrifugation and transfer the supernatant opalescent fluid to a fresh test tube. The specimen so prepared must be kept refrigerated if not tested at once.

Three small Kahn tubes are used for each test. It is well to use saliva from known secretors and from known nonsecretors as controls during the test. The controls are treated in the same manner as the unknown specimens.

1. In **tube 1** place 1 drop of the treated saliva. Add 1 drop of anti-**A** serum.
2. In **tube 2** place 1 drop of the treated saliva. Add 1 drop of anti-**A** serum.
3. In **tube 3** place 1 drop of the treated saliva. Add 1 drop of anti-**B** serum.
4. Allow to stand at room temperature for 1 hr.
5. To tube 1 add 1 drop of saline-suspended group A_1 cells.
6. To tube 2 add 1 drop of saline-suspended group A_2 cells.
7. To tube 3 add 1 drop of saline-suspended group B cells.
8. Allow to remain in contact for 30 min. at room temperature, shaking the tubes every 5 min. Observe macroscopically for agglutination or lack of agglutination. Confirm the readings by placing a small amount of sediment from each tube on a properly labeled glass slide and examine under a microscope, or observe the contents of the tubes under a scanning lens.

If the results in tube 1 or 2 are negative, the saliva contained group-specific substance A and was derived from a secretor of A substance.

If the result in tube 3 is negative, the saliva contained group-specific substance B and was derived from a secretor.

Saliva from nonsecretors will not inhibit the action of anti-**A** on group A cells nor of anti-**B** on group B cells. See Table 333.

Explanation: Agglutinogens present in saliva (or semen, etc.) will absorb or inhibit their corresponding agglutinins from known sera. The A agglutinogen will inhibit anti-**A**, which leaves anti-**A** serum inactive against group A cells. The B agglutinogen will inhibit anti-**B**, leaving the anti-**B** serum inactive against group B cells.

Use of anti-H lectin to detect secretors of O and A_2 substances

The discovery that extracts of seeds of *Ulex europaeus* gave agglutination reactions resembling those of anti-**O** serum has made

Table 333. *Results possible in inhibitory test for secretors*

Unknown treated saliva + *anti-***A** *serum* + *A_1 cells*	*Unknown treated saliva* + *anti-***A** *serum* + *A_2 cells* =	*Unknown treated saliva* + *anti-***B** *serum* + *B cells*	*Conclusions*
−	−		Secretor of A substance
+	+		Nonsecretor of A substance
		−	Secretor of B substance
		+	Nonsecretor of B substance

available a practicable method of testing for the secretor character.[187, 188] This reagent gives sharp reactions despite its low titer and can be used for testing saliva of individuals of any A-B-O group. It is not specific for blood group O since it tends to react most strongly not only with red cells from group O individuals but also with red cells from group A_2 persons. It is therefore called anti-**H** and not anti-**O**. (See pp. 1543 and 1620.)

Collect the saliva in dry, wide-mouthed test tubes. The individual being tested may chew on gum or paraffin to increase the flow of saliva. In drooling infants or in young babies the saliva can be scooped up into small test tubes. It may be necessary to collect the saliva from infants on cotton swabs by rubbing the swabs across the floor of the mouth and inside the cheeks until they are saturated. Then force the swabs into a small tube to express the saliva. Repeat the process until an adequate quantity is obtained.

As soon as the specimens are collected, place in a boiling water bath for 10 min. or bring to a boil over a Bunsen burner. Centrifuge the coagulated mass and transfer the supernatant opalescent fluid to a fresh test tube. Store in a refrigerator until ready to use.

Best results are obtained when the tests are made in serial dilutions. Prepare a 4-fold series of dilutions of the heated saliva as follows: undiluted, 1:4, 1:16, 1:64, and 1:256. (Method of preparing accurate dilutions is explained on p. 1476.)

1. Prepare 5 small Kahn tubes, inside diameter 7-8 mm., for each specimen and for (a) a specimen from a known secretor and (b) a specimen from a known nonsecretor.
2. Place 1 drop of saliva in its corresponding tube in the series (1 drop of undiluted specimen in tube 1, 1 drop of 1:4 in tube 2, etc.).
3. Add 1 drop of anti-**H** reagent to each tube.
4. Mix and allow to stand for 5 min. at room temperature.
5. Add to each tube in each series 1 drop of 2% suspension in saline of fresh, washed group O cells.
6. Shake the rack and place in a refrigerator for 2 hr. When the rack is removed from the refrigerator, place it on a tray of ice to keep the tubes cold while reading the results of the test. If no positive control saliva is available, use one of the commercial preparations of A-B blood group substance.

If the individual is a secretor of the H substance (O and A_2), the group O cells will not be agglutinated. If the individual is a

Table 334. *Example of inhibition test for factor H in saliva (O and/or A_2)**

Saliva specimen	*Reaction with group O cells of anti-H reagent to which saliva has been added in dilution*					*Diagnosis*
	1:1	*1:4*	*1:16*	*1:64*	*1:256*	
1.	–	–	–	Trace	++	Secretor
2.	–	–	Trace	++	++	Secretor
3.	++	++	++	++	++	Nonsecretor
4.	–	–	–	–	Trace	Secretor
5. Known secretor	–	–	–	–	++	
6. Known nonsecretor	++	++	++	++	++	
7. Commercial group substances	–	–	–	–	Trace	

*From Wiener, A. S.: Lab. Digest 22:1, July, 1958.

Table 335. *Example of inhibition tests with anti-H reagents against saliva samples from different individuals**

Specimen	*Blood group*	*Secretor status*	*Reaction with group O cells of mixture of anti-H reagent and boiled saliva diluted*					*Inhibition titer*
			Undiluted	*1:4*	*1:16*	*1:64*	*1:256*	
1	O	Secretor	–	–	–	–	+±	64
2	O	Secretor	–	–	–	Trace	++	32
3	A_1	Secretor	–	–	–	++	++±	16
4	O	Nonsecretor	++	++±	++±	+++	+++	0
5	B	Nonsecretor	++	++±	++±	++±	+++	0
6	A_1	Secretor	–	–	++	++±	++±	4
7	A_1	Nonsecretor	+++	+++	+++	+++	+++	0
8	O	Secretor	–	–	Trace	++	++	8
9	A_1	Secretor	–	Trace	++	++	++±	2
10	B	Secretor	–	–	++	++±	+++	4
11	A_1B	Secretor	–	–	Trace	++	++±	8
Reference standards	Commercial solution of blood group substances		–	–	–	–	+±	64
	Nonsecretor saliva		++±	+++	+++	+++	+++	0

*Reprinted from Wiener, A. S.: Journal of Forensic Sciences, Volume 3, No. 4, October, 1958, published by Callaghan & Company, Chicago, Ill.

nonsecretor, the cells will be agglutinated. Refer to Tables 334 and 335.

Recapitulation on Lewis blood groups

It has been known for some time that occasionally human sera contain naturally weak agglutinins that give reactions unrelated to the A-B-O system. Many of these remain unclassified. In view of the poor reactions given by these weak irregular agglutinins, which give the strongest clumping at low temperatures under conditions in which nonspecific cold agglutinins interfere, there was little interest in the natural irregular agglutinins until the **Lewis** factors were found. Two related **Lewis** factors have been found and are designated as **Le**^{a} and **Le**^{b}. It has been established that adults who are Le(a+) are almost invariably nonsecretors of the A-B-O substances, whereas Le(a–) persons are secretors. Many aspects of the **Lewis** blood factors, especially the relationship between **Le**^{a} and **Le**^{b}, are still unclear because of the difficulty of obtaining potent specific reagents. Although considerable difficulty has been encountered in carrying out tests for the **Lewis** factor, 3 well-defined Lewis types have been identified: Le(a+b–), Le(a–b+), and the rare Le(a–b–). In contrast to all other known blood factors, the **Le**^{a} factor appears to be hereditarily transmitted by a simple mendelian recessive.

Wiener has suggested that the **Le**^{a} substance is identical with the **T** substance found in nonsecretor saliva by Uyeyama and that **Le**^{b} substance is the same as or related to the **H** substance of secretor saliva, although some preparations of **H** substance have **Le**^{b} activity whereas others do not.

Division of human beings into 2 hereditary types,[189] depending on the presence or absence of blood group substances in the secretions and body fluids, is determined by identification of the **Le** factor. Those who are Le(a+) are nonsecretors, and those who are Le(a–) are secretors. For example, in group A individuals who are secretors, the blood group substance A is demonstrable in high concentration in the saliva and other secretions, whereas in nonsecretors of group A no blood group substance A is demonstrable. It has been suggested that the reason why most babies of group A or group B do not have erythroblastosis fetalis when their mothers are group O is that the babies are secretors, so that the maternal isoantibodies that reach their circulation are neutralized before they can combine with the baby's erythrocytes, and that only nonsecretors are affected. However, since the same high incidence of secretors has been found among babies with erythroblastosis fetalis as in the general population, this cannot be the correct explanation. A more plausible explanation is the incomplete development of the A-B-O agglutinogens in the newborn infant and the low avidity of the agglutination reactions of their red cells.

FACTORS P AND Tj^{a}—P-p SYSTEM

Landsteiner and Levine[190] in 1928 described an agglutinogen P in human blood, more frequently found in Negroes than in whites. They demonstrated this factor with immune rabbit serum. Occasionally a natural irregular anti-**P** agglutinin is found in human serum, which acts on the P agglutinogen in human cells. The reaction is extremely weak but may sometimes be demonstrated if the test is made in small tubes and not centrifuged but kept in a water bath at 20° C., shaken occasionally, and then examined under the microscope.

Anti-**P** agglutinins have been found in the sera of certain normal, nonimmune animals, such as rabbits, hogs, cats, and cattle. Factor **Q** of the Japanese investigators has proved to be the same as factor **P**.

Landsteiner and Levine concluded that the property P is not a single, well-defined entity but a group of related agglutinogens.

Occasionally anti-**P** agglutinins are found in normal human sera and are of sufficiently high titer to be usable as diagnostic reagents. Anti-**P** sera have been prepared from selected sera from P-negative professional donors containing an exceptionally high titer of natural anti-**P** agglutinins.

Tests for P agglutinogens are at times helpful in special problems of incompatibility in blood transfusions. The tests have also been used in anthropologic investigations and in serologic studies on individual differences in human blood.

The P agglutinogen is inherited as a simple mendelian dominant by a pair of allelic genes designated *P* and *p*, respectively. The tests can therefore be used as an additional factor in medicolegal cases of disputed parentage.

The names "type P" and "type pp" are used instead of the terms P positive and P negative.

There are also bloods containing a variant of the P agglutinogen.

Genotypes of P are *PP* and *Pp*; genotypes of pp are *pp*. Approximately 79% of Caucasoids and 98% of Negroids have been found to belong to type P, whereas 25% of Caucasoids and only 2% of Negroids have been found to belong to type pp. The percentage of type P among Mexican Indians has been found to be 78.9 and among Japanese, 30.

Most anti-**P** sera have the property of cold agglutinins for all human red cells. Removing the nonspecific antibodies by absorption weakens the anti-**P** agglutinins, making it difficult to prepare a serum to test for factor **P**.

Another type other than P positive and P negative was found by identifying an antibody in the serum of a blood donor. The factor detected by this serum was named **Jay** or **Tj**^{a} after the donor, Mrs. Jay. The anti-**Tj**^{a} serum agglutinated the red cells of 3000 consecutive random group O individuals but did not react with the red cells of the donor (Jay). Individuals who lack this factor are rare.

Factor **Tj**^{a} has been found to be related to **P**.

In the P system the following agglutinogens and their corresponding factors are found: P_1, containing factors **P** and **Tj**^{a}; P_2, with factor **Tj**^{a} only; and p, with no factors of this system.

Tests for factor P—Wiener–Unger

1. Prepare a saline suspension of the cells to be tested, wash once in saline, and resuspend to 3-5% concentration without enzyme treatment.
2. Place 1 drop of these cells in a small Kahn tube (7 mm. inside diameter).
3. Add 1 drop of anti-**P** serum.
4. Mix thoroughly and place in a refrigerator at 4° C. for 2 hr. Tilt the tube gently to dislodge the sediment and read the reactions macroscopically and under a scanning lens. Take care not to let the tube warm during the reading. Keep the rack containing the tubes in ice water or on a tray of ice until the readings have been made.

If the cells have agglutinated, they contain the **P** factor. If they fail to agglutinate, they do not contain the **P** factor. Do not make an antiglobulin test on cells when examining for the **P** factor.

See Table 336 for possible results.

Table 336. *Results possible in tests for factor* **P***

Unknown cells + *anti-***P** *serum*	*Phenotype*	*Genotype*
+	P	*PP* and *Pp*
−	pp	*pp*

*After A. S. Wiener, Brooklyn, N. Y.

Positive and negative control bloods should be included in the test, and the reactions in these tubes should be used for comparative reading of the unknown bloods.

Since the antibody is a cold agglutinin, the reactions will reverse if the tubes warm to room temperature. The proper temperature must be maintained throughout the test to avoid incorrect results. On humid days moisture sometimes condenses on the tubes. This moisture must be wiped off before the readings can be taken.

K-k SYSTEM—KELL-CELLANO TYPES

In 1946 Coombs et al.[191] found an antibody in the serum of a mother of an erythroblastotic infant which gave reactions unrelated to the Rh-Hr blood system. The factor defined by this antibody has been found in 7%[191] to 12.9%[192] of all persons tested. The blood factor was named **Kell**, after the patient from whom the serum was obtained. The factor **Si** in the serum of a patient who had had a hemolytic transfusion reaction described in 1947 by Wiener and Sonn-Gordon[192] proved to be identical to the **Kell** factor. In order to avoid confusion Wiener discarded the symbol **Si** for this blood factor which he had independently discovered and adopted the symbol **K** used by the British investigators.

Levine et al.[193] found a serum giving reciprocal reactions to anti-**K** in the serum of a mother of a mildly erythroblastotic infant. The antibody was called anti-**k** and the factor it defined, **k**. Additional examples of anti-**k** were found by Wiener and other investigators. The antibody found by Levine was derived from a patient named Cellano, and factor **k** is at times referred to as the **Cellano** factor. It has an incidence of 99.8% among Caucasoids.

When tests are made with both anti-**K** and anti-**k** sera, 3 types can be differentiated: type KK in 0.25%, type Kk in 9.5%, and type kk in 90.25% of Caucasoids.

Most of the anti-**K** sera must be used with the conglutination and antiglobulin technics. Limited quantities of anti-**K** serum are available commercially.

Testing for K factor—Wiener

Plasma conglutination method

Have on hand fresh oxalated plasma, either of group AB (no anti-**A** or anti-**B** agglutinins) or of a blood group compatible with the blood being tested. This is the supernatant liquid from whole blood taken in 3:2 dry oxalate (pp. 1462 and 1463).

1. Make a saline suspension of cells to be tested and wash once with saline; then resuspend to a 2% concentration.
2. Place 1 drop of washed saline-suspended unknown cells in a small tube (7-8 mm. inside diameter).
3. Add 1 drop of anti-**K** serum.
4. Mix and place in a water bath at 37° C. for 1 hr.
5. Remove the supernatant fluid with a fine capillary dropper **as completely as possible.**
6. Add 1 large drop of oxalated (AB) plasma and resuspend the sediment in it.
7. Place in a water bath at 37° C. for 1 hr.
8. Shake gently to dislodge the sedimented cells and read the reaction macroscopically and under a scanning lens.

If the cells have agglutinated, they are **K** positive. If they have not agglutinated, they are **K** negative.

Antiglobulin technic

1. Make a saline suspension of cells to be tested, wash once in saline, and resuspend in enough saline to make a 3% concentration.
2. Place 1 drop of this suspension in a small (7 mm. inside diameter) tube.
3. Add 1 drop of anti-**K** serum and mix.
4. Place in a water bath at 37° C. for 30-60 min.
5. Wash the mixture rapidly with saline 3 times, that is, fill the tube with saline, centrifuge, and decant the supernatant fluid; repeat 3 times. Remove and discard all the supernatant saline after the last washing, leaving only 1 drop.
6. Add 1 drop of antiglobulin (Coombs) serum and shake.
7. Centrifuge at low speed, 500 r.p.m., for 1 min.
8. Gently dislodge the sediment and read macroscopically and under a scanning lens.

If the cells have agglutinated, they contain factor **K**, but if they have not agglutinated, factor **K** is not present.

Testing for k factor

Since the anti-**k** serum contains a univalent antibody, best results are obtained by use of the antiglobulin technic.

1. Prepare a fresh suspension of the cells to be tested, wash once, and resuspend in saline to a 3% concentration.
2. Place 1 drop of the cell suspension in a small (7 mm. inside diameter) tube.
3. Add 1 drop of anti-**k** serum.
4. Mix and place in a water bath at 37° C. for 60 min.
5. Wash rapidly 3 times with saline (see above) and decant the saline after the last washing, leaving the cells suspended in 1 drop of the last saline.
6. Add 1 drop of antiglobulin (Coombs) serum and mix.
7. Centrifuge at 500 r.p.m. for 1 min.
8. Gently dislodge the sediment and read macroscopically and under a scanning lens.

Include as controls specimens of blood of known types KK, Kk, and kk with each test.

If the unknown cells have agglutinated, they contain factor **k**. If they have not agglutinated, they do not contain factor **k**. Refer to Table 337.

Table 337. *Results possible using anti-***K** *and anti-***k** *sera*

Reaction with			
*Anti-***K**	*Anti-***k**	*Phenotype*	*Corresponding genotype*
+	−	KK	*KK*
−	+	kk	*kk*
+	+	Kk	*Kk*
−	−	Error or rare atypical blood	

Factors Kp^a and Kp^b (Penney factor)

After the **K-k** factors had been found another antibody was discovered in the serum of an individual who had never been exposed to the antigen either through pregnancy or through transfusion. Two more sera of identical specificity were found later. The new factor determined by the antiserum was named **Penney** and it was later determined that it is related to the K-k system. This discovery gave rise to the identification of 2 factors, now designated as **Kp**^{a} and **Kp**^{b}.

Factor Ku

In 1961 Corcoran et al.[194] reported finding a new antibody, anti-**Ku** (anti-**Peltz**) in the Kell blood group system. Two unrelated families have been reported in which the Kell phenotype K negative, k negative, Kp^a negative, and Kp^b negative occurred.[195, 196] The propositus of the first family had in her serum antibodies that caused hemolytic disease in her fetus. Her serum has been found to contain an antibody which apparently is a new antibody in the Kell system, and it has been designated anti-**Ku**. It seems to detect an antigenic factor in the Kell system of nearly universal occurrence in human red cells. Successive adsorptions and elutions were performed using 3 bloods selected in such a way that the final eluate could contain no previously known blood group antibody. It agglutinated all of 525 bloods chosen at random and failed to agglutinate only very rare bloods which were of highly unusual Kell types. The letter "u" was selected because of the almost universal occurrence of the antigenic factor in human red cells.

Importance of K-k factors

Factors **K** and **k** are far less antigenic than is the **Rh$_0$** factor. As a cause of erythroblastosis fetalis, factor **K** ranks with factor **hr′** in importance. Tests for detecting the antibodies are very delicate and must be carried out with the greatest of care.

Anti-rh^w + K*

Serum anti-**rh^w+K** contains antibodies which detect the 2 blood factors **rh^w** and **K**. When this reagent is used for testing blood cells, it serves as a screening test for both factors. Both factors **rh^w** and **K** are of low frequency in the general population, and a great majority of bloods lack both. Such bloods are quickly identified by their negative reactions with this antiserum. Only the small percentage of positively reacting bloods need be further tested to determine whether they contain factor **rh^w** or **K** or both.

The tests are carried out by the saline agglutination and antiglobulin technics. A negative test therefore demonstrates that the unknown cells are not coated by antibodies, and this takes the place of the direct antiglobulin (Coombs) test.

If the reaction is positive, however, the unknown cells may already have been coated before this test was made. In such cases a direct antiglobulin test should be made of the cells. If such test is positive, it is usually not possible to determine whether or not factor **K** is present on the cells.

The **rh^w** antibodies in the anti-**rh^w+K** serum will clump cells containing the **rh^w** factor in saline suspension, whereas the **K** antibody reacts only by the conglutination and antiglobulin technics. The antiglobulin technic, which detects the **K** factor, also serves to improve the reactions for factor **rh^w**, and this test will also be positive, as previously stated, if the cells are coated by antibodies.

The method is of limited value when testing blood from patients known to have acquired hemolytic anemia or erythroblastosis fetalis because in such patients the cells are coated with antibodies and therefore give a positive direct Coombs reaction.

Procedure:

1. Prepare a cell suspension in saline, wash once, and resuspend the cells to a 2 or 3% concentration in saline.
2. Place 1 drop of the cell suspension in a tube (7 mm. inside diameter).
3. Add 1 drop of anti-**rh^w+K** serum.
4. Mix and place for 1 hr. in a water bath at 37° C.
5. Gently dislodge the sediment and read the reactions macroscopically and under a scanning lens.
6. Centrifuge at 500 r.p.m. for 1 min. and read again.

Clumping is a positive reaction and lack of clumping is a negative reaction.

Test all negatively reacting bloods by the antiglobulin technic:

1. Wash the sediment 3 times with large volumes of saline (fill the tube to the top with saline and mix the cells with the diluent, centrifuge, decant, and repeat 3 times).
2. After the last washing remove all but 1 drop of saline and resuspend the cells in this drop.
3. Add 1 drop of antiglobulin (Coombs) serum.
4. Centrifuge for 1 min. at 500 r.p.m.
5. Gently dislodge the sediment and read. See Table 338.

The vast majority of bloods will react negatively in both the saline agglutination and the antiglobulin methods. Such bloods lack both factors **rh^w** and **K**, and at the same time the negative reactions in the second stage of the test show that the unknown cells are not coated by antibodies.

If the reactions are negative in the first stage of the test but positive in the second stage, this indicates absence of the **rh^w** factor and the probable presence of the **K** factor. If the direct antiglobulin test is negative and tests with pure anti-**K** serum are positive, the presence of **K** is confirmed. If, on the other hand, the direct antiglobulin test is positive, it is generally not possible to determine presence or absence of factor **K** with any degree of reliability.

If the reactions are positive in the first stage of the test, they will also be positive in the second stage, demonstrating the presence of the **rh^w** factor. This can be confirmed, if required, by tests with pure anti-**rh^w** serum.

Since factors **rh^w** and **K** are inherited as simple mendelian dominants, theoretically this test could be used occasionally in cases of disputed paternity, but the number of investigations already carried out on blood factors **rh^w** and **K** is limited and the reagents available are of only moderate titer. The application of these tests in medicolegal cases, there-

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*From a pamphlet published by A. S. Wiener, Brooklyn, N. Y.

Table 338. *Results possible with anti-***rh**^{w}**+K** serum

*Anti-***rh**^{w}**+K** *serum* + *saline suspended unknown cells*	*Antiglobulin method*	*Interpretation*
−	−	Great majority of bloods give these reactions, which indicate that blood belongs to type kk and is **rh**^{w} negative
−	+	Indicates absence of **rh**^{w} factor; to test for factor **K** proceed as shown below
+	+	Indicates presence of factor **rh**^{w}; to test for factor **K** proceed as shown below

*Pure anti-***K** *serum plus unknown cells by antiglobulin method*	*Unknown cells plus antiglobulin serum—direct test*	*Interpretation*
+	−	Unknown blood contains **K** factor
+	+	Unknown cells are coated; tests for **K** factor are inconclusive

fore, is not recommended at the present time.[192, 197-200]

F-f SYSTEM—DUFFY OR F FACTORS

In February, 1950, Cutbush et al.[201, 202] and van Loghem and van der Hart[203] independently reported the discovery of a new blood factor. This new factor was not part of the A-B-O, M-N, P, or Rh-Hr systems or of other previously described systems. The antibody was found in the blood serum of a hemophiliac who had had a long series of blood transfusions over a period of 20 yr. and who sustained a mild transfusion reaction after the last injection of blood. Since this patient's name was Duffy, the antibody was designated anti-**Duffy**, and the factor it identified was called the **Duffy** factor. Other examples of **Duffy** sensitization were later reported by Ikin et al.,[204] Rosenfield et al.,[205] and Wiener.[206]

The **Duffy** factor is important in hemolytic transfusion reactions when **Duffy**-positive blood is transfused into **Duffy**-negative patients and in some of the puzzling cases of erythroblastosis fetalis not caused by other factors.

The symbol **Fy** was introduced by Cutbush and Mollison to indicate the **Duffy** factor. Those with the factor were originally called Fy(a+) and those who lacked the factor, Fy(a−). It was later found that the **Duffy** factor is inherited as a simple mendelian dominant by a pair of allelic genes, designated Fy^{a} and Fy^{b}. More recently Wiener has shortened the symbols to **F** and **f** for the factors and *F* and *f* for the genes.

All anti-**F** sera so far produced have been usable only by the antiglobulin method.[207] When the red cells are already coated with antibody, as in autohemolytic anemia or erythroblastosis fetalis, tests for the **F-f** factors cannot be carried out reliably. The tests are not made routinely in the usual hospital laboratory since identification of the factor is difficult. With the production of more reliable anti-**F** serum these tests no doubt will be added to the hospital routine.

Determining F-f factors

1. Make a suspension of the cells in saline, wash once in the usual manner, and resuspend to a 3% concentration in saline.
2. Place 1 drop of the 3% suspension of cells in a small tube (7 mm. inside diameter).
3. Add 1 drop of anti-**F** (**Fy**^{a}) serum.
4. Mix and place in a water bath at 37° C. for 30-60 min.
5. Wash rapidly with saline 3 times. Leave 1 drop of saline on the cells after the last washing. Resuspend the cells in this drop of saline.
6. Add 1 drop of antiglobulin serum (Coombs) and mix.
7. Centrifuge at 500 r.p.m. for 1 min.
8. Gently dislodge the sediment and read macroscopically and under a scanning lens.

Always include positive and negative controls. The washing with saline must be carried out rapidly to prevent elution of the antibody from the cells, with resulting false negative reactions. In addition, the red cells are fragile and may undergo

Table 339. *Results possible in F-f tests*

Unknown cells + *anti-***F** *serum*	*Phenotype*	*Genotype*
+	Fy(a+)	*FF* and *Ff*
−	Fy(a−)	*ff*

spontaneous clumping or hemolysis if kept in saline suspension for too long a time, another reason for washing rapidly.

M-N-S-U SYSTEM

Agglutinogens M and N

In 1927 Landsteiner and Levine[190, 208] discovered the M and N types when they immunized rabbits with human red blood cells. When the antisera thus produced by the rabbits were absorbed with certain samples of human blood, they still contained agglutinins acting on the majority of bloods of all 4 groups, although some of the bloods were not agglutinated. The antibodies produced by the rabbits were designated as anti-**M** and anti-**N**, respectively, and the factors they identified as **M** and **N**. The immune rabbit sera could not be used directly because they contained antibodies reactive for all human cells and therefore had first to be diluted and absorbed with human cells lacking the blood factor in question. The original method of immunizing rabbits is still used at the present time.

The anti-**M** and anti-**N** reagents define 3 types of human blood—M, N, and MN—with approximate frequencies among Caucasoids of 30%, 20%, and 50%, respectively. Type M contains agglutinogen M; type N, only agglutinogen N; and type MN, both agglutinogens M and N.

It has been proved by family studies that the 3 M-N types are transmitted by a pair of allelic genes, designated *M* and *N*, respectively. There are thus 3 genotypes corresponding to the 3 phenotypes: type M, genotype *MM*; type N, genotype *NN*; type MN, genotype *MN*. Because of the clear-cut heredity of the M-N types, the M-N tests find frequent application in medicolegal cases of disputed parentage and are also helpful in twin studies to determine whether a pair of twins of the same sex are monozygous or dizygous.

Agglutinogens M and N are but weakly antigenic in man and are ordinarily disregarded when selecting donors for blood transfusion. They are not as important as the A-B-O and Rh-Hr types for clinical medicine, but nevertheless anti-**M** and anti-**N** do occasionally occur in human sera, and when they do, they can give rise to hemolytic transfusion reactions. If present in the serum of a pregnant woman, this can lead to hemolytic disease in her newborn baby.

Wiener has noted that in the majority of transfusions donor and recipient belong to different M-N types, and the **M** and **N** factors are then useful as natural tags to trace the fate of the donor's cells in the patient's circulation. Such survival tests (designated "differential agglutination" by Wiener) are helpful in the study of transfusion reactions and in comparing the relative merits of different methods of storing blood in blood banks.

Only rarely have human sera been found to contain natural anti-**M** agglutinins, and natural anti-**N** is found even less frequently in human beings.* Although some cases of isoimmunization to factor **M** have been reported, the **M** and **N** factors are most important medicolegally in cases of disputed paternity. It is generally considered that agglutinogens M and N can be disregarded when selecting donors for blood transfusion therapy, but if a patient requires multiple transfusions over a period of months or years, the possibility of isosensitization to the M agglutinogen must be seriously considered.[209]

Bloods lacking both agglutinogens have not been found in tests on many millions of blood specimens, although variants and subtypes have been demonstrated.

Wiener and Forer[210] reported a case of **M** sensitization in an individual of type N in 1941, and Broman reported another case in 1944.[211] In 1948 Wiener et al.[212] indicated that sensitivity to agglutinogen M could be an important source of hemolytic reactions for type N individuals who received a series of blood transfusions when type M blood was injected. In 1950 Wiener[213] described a hemolytic transfusion reaction due to sensitization to the **M** factor in a type N patient.

It is interesting to note that whereas the **M** and **N** factors are seldom antigenic to man, they are highly antigenic to rabbits.

Factors and subtypes of agglutinogen M

(1) **$\mathbf{M_i}$, $\mathbf{M_{ii}}$, $\mathbf{M_{iii}}$, $\mathbf{M_{iv}}$, etc.:** As Wiener has pointed out,[214] not all anti-**M** agglutinins are identical in specificity, showing that there are more than one kind of **M** factor. If the agglutinin which reacts with human red cells but not with blood from chimpanzees or monkeys is designated as anti-$\mathbf{M_i}$, that which detects a factor common to both human M blood and chimpanzee blood but absent from the blood of the lower primates as anti-$\mathbf{M_{ii}}$, etc., then human M agglutinogen has all of the blood factors $\mathbf{M_i}$, $\mathbf{M_{ii}}$, $\mathbf{M_{iii}}$, $\mathbf{M_{iv}}$, etc., chimpanzee blood has factors $\mathbf{M_{ii}}$ and $\mathbf{M_{iii}}$, etc.

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*A report by Masters and Vas[208a] discusses anti-**N** formed by a 10-week-old infant.

The discovery of the Rh factor came about when Landsteiner and Wiener prepared anti-**M** reagents by injecting the blood of rhesus monkeys into rabbits for the purpose of producing anti-**M** reagents which might be specific for human M cells.

(2) **Subtype $\mathbf{M_2}$:** Friedenreich and Lauridsen[215] reported a weak form of the **M** factor which differs qualitatively from ordinary **M**. Red cells carrying the antigen were agglutinated by 5 out of 9 rabbit anti-**M** sera and by 1 out of 4 human anti-**M** sera.

(3) **Factor $\mathbf{M^c}$:** Dunsford et al.[216] described an antigen, called by them $\mathbf{M^c}$, which reacted with 25 out of 27 anti-**M** and with 5 out of 28 anti-**N** sera; they gave it the designation $\mathbf{M^c}$. The antigen has been found in only one family. It is considered to be intermediate between **M** and **N**.

(4) **Factor $\mathbf{M^g}$:** Allen et al.[217] found a very rare antigen, which they called $\mathbf{M^g}$, in one selected family. The antibody, anti-$\mathbf{M^g}$, has been found in 4 out of 500 normal people tested. It reacts with cells suspended in saline, and the reaction is rendered easier to read if the mixture of cells and serum are incubated for 5 min. at 37° C. and then centrifuged.

The gene for antigen $\mathbf{M^g}$ appears to be allelic to genes *M* and *N*. Blood determined by the genotype M^gM, if tested only with anti-**M** and anti-**N** sera, gives the reaction of type M blood. Blood representing genotype M^gN, tested only with anti-**M** and anti-**N** sera, appears to be N.

(5) **Factors $\mathbf{Mi^a}$ and Vw:** Levine et al.[218] found an antibody as the cause of hemolytic disease in the husband and 2 of the children of the propositus but not in 425 random group O and group A Caucasoids tested. They called the antibody anti-$\mathbf{Mi^a}$ and the factor it identified, $\mathbf{Mi^a}$.

Later, van der Hart et al.[219] described an antibody, anti-**Vw**, coating the cells of a baby. The direct antiglobulin test was positive. It was believed at first that **Vw** and $\mathbf{Mi^a}$ were identical, but later it was found that half the $\mathbf{Mi^a}$-positive persons were **Vw** positive and half were **Vw** negative. No $\mathbf{Mi^a}$-negative **Vw**-positive person has been found to date.

These 2 factors have been found to be related to the M-N-S-U system.

(6) **Factor $\mathbf{M^e}$:** Wiener and Rosenfield[220] in 1961 observed a discrepancy in the reactions obtained when using 2 different anti-**M** reagents to test the blood cells of a Negro involved in a medicolegal case of disputed parentage. Both reagents had been prepared in the usual manner by injecting rabbits with type M cells and absorbing the diluted serum with type N cells until they no longer agglutinated the absorbing cells (p. 1555). Further tests showed that the blood cells which gave the conflicting reactions had come from a type N individual who was He (Henshaw) positive (p. 1554), although the immunized rabbits had almost surely not received He-positive cells in error. These findings were similar to those of Mourant and associates which led to the discovery of the **He** antigen.

The anti-**M** reagent had been believed to be ordinary anti-**M** serum until the individual of type N.He was encountered. Five additional unrelated type N.He bloods were also agglutinated by this antiserum, but all other bloods of type N which were Henshaw negative, even if Hu (Hunter) positive, were not agglutinated.

When this anti-**M** fluid was absorbed with type M cells, it lost all its agglutinating activity, including that for type N.He (type N, He positive) red cells. Absorption with an equal amount of type N.He cells removed the reactivity for the absorbing cells but did leave some weak residual activity for cells having the **M** antigen, which could be removed by additional absorption with N.He cells. Thus this anti-**M** reagent presumably contained a single antibody for a blood factor shared by cells having the antigen **M** and cells of type N.He. Wiener and Rosenfield proposed the designation of this blood factor as $\mathbf{M^e}$ and the corresponding antibody as anti-$\mathbf{M^e}$.

The blood factor $\mathbf{M^e}$ appears to be shared by the **N** and **He** antigens and may be a source of error in M-N tests. If the putative father and the child in a case of disputed paternity are both type N, He positive, and the mother's type is N, He negative, a false exclusion of paternity can result if the individuals involved are tested at different times with different reagents. Thus, if the putative father is tested with ordinary anti-**M** and the child with anti-$\mathbf{M^e}$, the types of the 2 putative parents would be reported as N and that of the child as MN, with a resulting false exclusion of paternity.

Factors and subtypes of agglutinogen N

(1) $\mathbf{N_i}$ **and** $\mathbf{N_{ii}}$**, etc.:** As Wiener has shown, agglutinogen N also has a mosaic structure, just as M has.[221] Only 2 factors have been demonstrated so far—$\mathbf{N_i}$, which is peculiar to

human N cells alone, and $\mathbf{N_{ii}}$, which is shared by human N cells and chimpanzee red cells. When a human being inherits an M or N agglutinogen, this carries with it all of the serologic factors that characterize each agglutinogen.

(2) **Subtype N_2**: In 1935 Crome described a subtype of type N called N_2.[222] This type of blood is characterized by its very weak reactivity with anti-N sera. With many anti-**N** reagents, clumping may fail to occur at all or may be so weak as to be overlooked. In medicolegal cases this may lead to serious errors. It is always good practice to check all N-negative bloods with more than one anti-**N** serum of different lot numbers and from different manufacturing sources to prevent errors that might arise in this subtype.

Serology of M-N blood types

In parallel tests different anti-**M** and anti-**N** reagents generally give identical results with human red cells, but when the blood of lower primates is tested, the reagents often give different results, showing that these reagents possess different serologic specificities. There are at least 5 different **M** factors and at least 2 different **N** factors, as just explained. Since all human bloods with M agglutinogen have all the **M** factors and all N bloods have all the **N** factors, this can be disregarded when testing human blood because all anti-**M** and anti-**N** reagents necessarily will give the same results. An exception to this rule is the recently discovered anti-$\mathbf{M}^e$ (p. 1551), which gives strong reactions with blood from the rare type N individuals (most often Negroes) who have the **He** factor. Obviously, the unwitting use of anti-$\mathbf{M}^e$ reagents instead of standard anti-**M** can lead to the erroneous classification of such type N persons as type MN.

Ottensooser made the remarkable demonstration that extracts of seeds of *Vicia graminae* give reactions paralleling anti-**N** in specificity. Such seed extracts make excellent anti-**N** reagents but have not found wide practical application to date because of their limited availability.

Although it is possible to prepare anti-**M** reagents of very high titer, up to 256 units or higher, specific anti-**N** reagents, even though of excellent avidity, seldom exceed a titer of 10 units. The reason is that type M blood, in addition to the **M** factors, also has a weakly reacting **N**-like blood factor. For this reason, when anti-**N** immune rabbit serum is absorbed by type M blood to remove the species agglutinins, the desired anti-**N** agglutinin is weakened at the same time, whereas underabsorption will result in a nonspecific reagent. This applies to anti-**N** reagents prepared from *V. graminae* seeds as well as to anti-N rabbit or human serum. The presence of **N**-like factors in human type M cells explains why anti-**N** occurs so very rarely in human serum. Anti-**M** occurs less rarely; Wiener, for example, has observed more than a score of human sera with anti-**M** but has observed only a single human serum with anti-**N**. See footnote, p. 1550.

The M-N system, which at first was believed to be a simple system of only 3 types, has now become almost as complex as the Rh-Hr system. This has been due to the discoveries of such factors as **S, s, U, He, Hu, Vw, Mi,** $\mathbf{M}^g$, and $\mathbf{M}^e$.

Factors S and s of M-N-S-U system

Sanger et al.[223] in 1947 found in the serum of a mother of an erythroblastotic baby a blood group antibody that gave reactions different from any previously described.[224] The patient had been isosensitized to the $\mathbf{Rh_o}$ factor and also had $\mathbf{Rh_o}$ antibodies in the serum. Sanger and Race[223] were able to show the relationship between the factor detected by this antibody and the M and N agglutinogens. The factor determined by this antibody was designated **S** by Sanger and Race.

When persons of types M, MN, and N were subdivided according to their reactions with anti-**S** serum, 6 types of blood could be distinguished. The presence of **S** was noted by the use of the large letter "S" and its absence by the small letter "s." The 6 types are M.S, M.s, N.S, N.s, MN.S, and MN.s. The incidence of the **S** factor is not the same in the 3 M-N types, for whereas ⅔ of type M individuals are S positive, only about ⅓ of type N individuals are S positive.

When **S** is considered alone, 2 types are distinguishable, S+ and S–. S+ has a frequency of 56.1% and S– has a frequency of 43.9% in tests made on 394 donors in New York City and cited by Wiener.[214]

In 1951 Levine[225] found a serum of a mother of an erythroblastotic infant which gave reactions reciprocally related to anti-**S** and corresponding to those expected for factor **s**. Additional examples of this antiserum were subsequently found by other workers. Although a number of satisfactory anti-**S** sera have been found, sera of specificity anti-s are in very short supply and are more or less of a serologic curiosity.

To date there are 9 phenotypes recognized in the M-N-S system. This does not consider the factor **U** or any of the other factors to be discussed in the following pages. These 9 phenotypes are M.SS, M.Ss, M.ss, N.SS, N.Ss, N.ss, MN.SS, MN.Ss, and MN.ss. The factor **S** is a simple mendelian dominant. When factor **S** is associated with blood factor **M** in a parent, it is similarly associated in his children and in other members of his family. This is also true of factor **S** when it is associated with factor **N.** Factors **S** and **M** are not properties of different substances but are properties of one and the same agglutinogen, which supports the theory advanced by Wiener of multiple alleles in preference to that of linked genes.

Blood factor U

In 1953 Wiener et al.[226] found in the serum of a Negroid woman an abnormal antibody which strongly agglutinated the cells of the 2 donors whose blood had been used in transfusing her. Both of the donors belonged to group B MN rh kk, whereas the patient was group B MN $\mathfrak{R}h_1$rh kk. The patient had received Rh-negative blood because she had been erroneously typed as Rh negative, but this error in typing had no bearing on the hemolytic transfusion reaction reported by these authors.

This was a case of a fatal hemolytic reaction in a woman who entered the hospital with a diagnosis of bleeding peptic ulcer. She was given a transfusion, but this was discontinued after 100 ml. blood had been administered because of a reaction consisting of chills and fever. Another blood transfusion was carried out a week later. The patient died shortly thereafter.

The antibody found in the serum was of high titer and avidity. At body temperature the titer was approximately 400 units, and at refrigerator temperature it was only 40 units. This antibody was active by ordinary agglutination method in saline medium, and in this case it was a bivalent antibody. The strength of the reaction was not affected by proteolytic enzymes. In the original series of tests on 690 Caucasoids and 425 Negroids, no negative reactions with this serum were found among the Caucasoids, and only 4 negatively reacting persons were encountered among the Negroids. The factor detected by this patient's serum was called **U** to designate its almost universal nature.

In a further series of tests[227] of 1100 Caucasoids and 989 Negroids, no Caucasoids were found lacking factor **U**, and only 12 Negroids lacked the factor. Wiener et al.[227] showed the possible relationship of factor **U** to the M-N system. From family studies in the rare individuals of type uu (those who lack the factor), it was postulated that the factor is inherited by a pair of allelic genes *U* and *u*, *U* determining presence of the factor and *u*, its absence.

Although Wiener et al. showed that all U-negative individuals tested by them were either type N or type MN, Sanger et al.[228] found that all blood specimens that were **U** negative also lacked both blood factors **S** and **s**. Since it had previously been shown that factors **S** and s and blood factors **M** and **N** are associated with one another, this proved that blood factor **U** belongs in the M-N-S system.

In a series of tests on another sensitized patient, Wiener and Unger demonstrated that the **U** antibodies failed to clump saline-suspended ficinated cells, pointing to a relationship to the M-N-S system, because **M, N,** and **S** antibodies, in contrast to the Rh-Hr antibodies, generally fail to clump saline-suspended ficinated cells containing the specific blood factor. They also showed, in this patient, the presence of univalent **U** antibodies.

Wiener and Unger made an interesting observation regarding the number of antigenic sites per red cell in the M-N-S-U and the Rh-Hr systems.[229] They found a similar ratio between the antiglobulin titer and the blocking titer for **U** antibodies and for $\mathbf{Rh_0}$ antibodies suggesting that the number of antigenic sites per red cell are approximately equal for the 2 systems. Wiener and Unger also showed that there is evidence that the number of antigenic sites on the red cell surface for the **F** (**Duffy**) and **K** (**Kell**) factors is lower than for the M-N-S-U and Rh-Hr systems.[229]

Hu (Hunter) factor

In 1934 Landsteiner et al.[230] injected the blood of a Negroid (Mr. Hunter) into rabbits and obtained an antiserum that agglutinated the blood cells of 7.3% of Negroids and 0.5% of whites. The antigen was also found on the cells of 22% of West Africans; it is rare in Europeans. Wiener[231] found that all of the blood specimens that gave distinct positive reactions with this antiserum were type N or type MN, which suggested a relationship to the M-N system. Isoimmune antibodies in man have not as yet been reported.

He (Henshaw) factor

In an attempt to repeat the experiments which had resulted in finding the **Hu** factor, Chalmers et al.[232] injected the blood of a Nigerian (Mr. Henshaw) into a rabbit, with the resultant production of anti-**He** antibodies by the rabbit. The factor identified by the antiserum, **He**, has been found in 38 of 1390 West Africans or 2.7%, but it was not found in the blood of the 1500 Europeans tested. When most of the bloods positive for the **He** factor were found to have antigens **N** and **S** as well, it was shown to belong to the M-N-S system. Comparative tests with the original anti-**Hu** serum showed anti-**Hu** and anti-**He** to be different.

When **Hu** or **He** is associated with factor **M** in the parents, there is a similar association in the children. As Wiener and Wexler pointed out,[214] this indicates that **Hu** and **He** do not represent attributes of separate substances but together with **M, N, S,** s, and **U,** as the case may be, are factors of one and the same agglutinogen. No natural or isoimmune antibodies of the nature **He** have been found to date in human beings. See M^e, p. 1551.

Tests for M and N factors in blood cells

Blood for M and N tests may be collected in dry tubes such as those used in the Wassermann test, in tubes containing dried oxalate powder (p. 1463), or by mixing the blood with ACD* solution in the proportion of 4 parts blood to 1 part ACD. Venous blood is preferred, and blood taken in dry oxalate is most satisfactory not only for the M-N tests but also for the A-B-O and Rh tests. In infants or in patients with small or inaccessible veins, an adequate sample for blood grouping tests can usually be obtained from a deep puncture of the heel or of one of the fingers, after the hand has been immersed in hot water to produce hyperemia. Finger blood is collected in small tubes, inside diameter 7-8 mm., containing a few drops of ACD solution, in the proportion of 5-10 drops blood to 1 drop ACD solution.

Procedure:

1. Prepare a fresh 2% cell suspension in saline of the unknown bloods.
2. Using a welled or ceramic slide, place 1 drop of blood cell suspension to be tested in each of 2 wells.
3. Add 1 drop of anti-**M** serum to the left-hand ring and 1 drop of anti-**N** serum to the right-hand ring. The drops should fill the wells.
4. Place the slide on a rotating machine or rotate by hand for 10 min.
5. Take readings macroscopically and under the low power of a microscope and interpret as in Table 340.

In positive reactions the clumps are large and easily visible to the naked eye, whereas microscopically the agglutination is complete with few free cells between the clumps.

In negative reactions the suspension remains homogeneous, and upon microscopic examination each cell is separate and distinct.

Occasionally bloods will show partial agglutination, that is, large clumps on a background of free cells; in all such cases it should be possible to elicit a history of recent blood transfusion, showing a mixture of 2 different M and N types of cells in the individual's circulation.

Controls: Controls are made at the same time as the test, using known M, N, and MN cell suspensions against the anti-**M** and anti-**N** sera. These are handled just like the tests. Possible results are given in Table 341.

If any other results are obtained with the controls, the cause must be determined and new antisera tested. The unknown bloods then should be tested with the new antisera.

Sources of error in M and N testing technic: False negative reactions may result when old blood suspensions are tested, particularly when using anti-**N** serum. This is avoided by using only fresh blood.

False positive reactions may occur if the tests are held too long and drying occurs.

Anti-**M** serum agglutinates all blood containing the M agglutinogen (types M and MN), whereas anti-**N** serum agglutinates all

Table 340. *Results possible in M-N tests*

Reactions with			
*Anti-***M** *serum*	*Anti-***N** *serum*	*Blood belongs to*	*Approximate frequency (%) among Caucasoids*
+	−	Type M	30
−	+	Type N	20
+	+	Type MN	50
−	−	Regarded as an error	

Table 341. *Results expected in the controls*

Cells	*Anti-***M** *serum*	*Anti-***N** *serum*
M	+	−
N	−	+
MN	+	+

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*ACD (acid-citrate-dextrose solution) is made by dissolving 2.2 gm. trisodium citrate (dihydrate), 0.8 gm. citric acid (monohydrate) and 2.45 gm. dextrose (anhydrous) in 100 ml. distilled water. Use 75 ml. for 500 ml. blood.

Table 342. *Heredity of M-N types*

Blood types of parents	*% of children of* Type M	Type N	Type MN	*Parentage excluded if child belongs to type*
M × M	100	0	0	N or MN
N × N	0	100	0	M or MN
M × N	0	0	100	M or N
M × MN	50	0	50	N
N × MN	0	50	50	M
MN × MN	25	25	50	—

blood containing the N agglutinogen (types N and MN). To date no proved case has been found with blood not reacting with either anti-**M** or anti-**N** serum. Note that, theoretically, if the hypothetical M^gM^g individual exists and is ever found, such blood may fail to react with both anti-**M** and anti-**N** sera.

Production of anti-M and anti-N sera—Wiener[231, 233]

In order to prepare anti-**M** and anti-**N** sera by immunizing rabbits, it is necessary to procure a number of types OM* and ON donors. All sterile precautions must be observed throughout the procedure.

1. Withdraw 50 ml. type OM and 50 ml. type ON blood directly into their respective 50 ml. Rockefeller centrifuge tubes, each containing 4 ml. of sterile 3.8% sodium citrate solution. Shake to prevent clotting. It is best to pool blood from several OM donors and to do likewise with blood of several ON donors.
2. Wash with sterile saline several times until all plasma has been removed. This prevents possible anaphylaxis and production of antibodies for human serum globulin in the rabbits. Restore to original volume.
3. Add 12 ml. of this blood to 48 ml. saline to provide sufficient material to inject 4 rabbits 6 times each. Keep the blood refrigerated when not in use.
4. Use 4 rabbits† for anti-**M** and 4 for anti-**N** production. Each rabbit must be identified with an ear tag, and complete records must be kept. Inject 2 ml. OM blood into the marginal ear vein of each anti-**M** rabbit and 2 ml. ON blood into the marginal ear vein of each anti-**N** rabbit.
5. Repeat the injections daily for 6 days.
6. Allow 1 wk. rest interval; then repeat the 6 injections, giving the first intramuscularly and the other 5 intravenously. Giving the first injection intramuscularly prevents anaphylaxis and possible agglutination of the injected cells and death of the rabbit.
7. Allow another rest interval of 1 wk.; then repeat the 6 injections, giving the first injection intramuscularly and the other 5 intravenously.
8. Wait another week and test to determine whether anti-**M** or anti-**N** agglutinins have been produced in any of the animals. The entire period of injections and intervals covers 6 wk.

Tests must be made of the serum of each injected rabbit before withdrawing all its blood to be used as a laboratory reagent.

Preliminary tests of immunized rabbit serum for M and N antibodies:

1. Withdraw 2 ml. blood from the ear vein of each rabbit; place in a clean, dry, sterile Kahn tube properly labeled with the type letter and the identifying number of the rabbit from which the blood has been withdrawn.
2. Allow to clot, loosen the clot, centrifuge, remove the supernatant serum, and place it in a clean, dry, sterile Kahn tube properly labeled.
3. Inactivate the serum for 30 min. at 56° C. to destroy the complement and prevent subsequent hemolysis.

Since the sera of immunized rabbits contain species agglutinins, it is necessary to remove these before any tests can be performed. These are removed by absorption.

To absorb anti-**M** serum, pooled groups ON, AN, and BN cells are added to the rabbit serum. To absorb anti-**N** serum, pooled groups OM, AM, and BM cells are added to the rabbit serum. The anti-**M** serum is not easily overabsorbed, but the anti-**N** serum is, and therefore great care must be used in absorbing anti-**N** serum.

Absorptions are usually carried out at room temperature, using sufficient quantities of absorbing cells to ensure complete removal of the species antibodies. If the absorptions are carried out at lower temperatures, smaller amounts of blood cells are required.

1. Dilute the antiserum 1:20 by adding 2 drops of serum to 38 drops of saline.
2. Place 1 ml. diluted serum in a Kahn tube.
3. Add 0.5 ml. of washed cells of correct specificity to the serum. If preparing anti-**M** serum, use N cells; if preparing anti-**N** serum, use M cells.
4. Mix thoroughly, allow to stand 30 min. at room temperature, centrifuge, and use the supernatant fluid. In preparing anti-**N** serum,

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*Group O. type M.

†Four rabbits are used because some might fail to develop sufficient antibodies.

place the tube in a glass of warm water in an incubator at 37° C. for 30 min., then centrifuge, and use the supernatant fluid.

5. Test each serum against several specimens of M, N, and MN bloods.
6. On a slide containing 3 wax rings, properly labeled, place 1 drop of type M cells in the first ring, 1 drop of type N cells in the second ring, and 1 drop of type MN cells in the third ring. These are 2% suspensions in saline.
7. Add 1 drop of anti-**M** testing fluid to each ring.
8. Rotate and examine periodically under the low power of a microscope for 7-10 min.
9. Prepare a similar slide, using anti-**N** instead of anti-**M** testing fluid.

If the anti-**M** fluid agglutinates all M and MN cells within 1 or 2 min. but fails to agglutinate N cells after 10 min., it is then titrated for antibody content.

If the anti-**N** fluid agglutinates all N and MN cells within 1 or 2 min. but fails to agglutinate M cells within 7-10 min., it is then titrated for antibody content.

If the anti-**M** fluid agglutinates N cells or if the anti-**N** serum agglutinates M cells, the serum must be reabsorbed, care being taken not to overabsorb. If the **anti-**M serum now fails to agglutinate M cells or if the anti-**N** serum fails to agglutinate N cells, the serum cannot be used.

Titrate each fluid, using the technic on p. 1476. The anti-**M** fluid should have a minimum titer of 10-20 units and the anti-**N** fluid a minimum titer of 5-10 units.

Collection of anti-M and anti-N testing fluids: Having selected the rabbits that show agglutinin anti-**M** in a titer of at least 10 and anti-**N** in a titer of at least 5-10, exsanguinate by severing the carotid artery, using utmost sterile precautions, and obtaining the entire content of blood from each animal. For the method of bleeding rabbits from the carotid artery, refer to p. 808.

1. Collect the blood in clean, dry, sterile 50 ml. Rockefeller centrifuge tubes, properly labeled. Cork immediately and allow to clot with the tubes in a slanting position.
2. Refrigerate for 24 hr., strike the tubes against the palm of the hand to loosen the clot, and then centrifuge at high speed for 15-30 min. to separate the serum from the clot.
3. Under sterile conditions withdraw the serum and place it in sterile 20 ml. test tubes, properly labeled and stoppered with cotton.
4. Inactivate at 56° C. for 30 min.
5. Retain a few ml. for absorption.
6. Ampule the remaining serum, labeling with the rabbit's identification number, the type letter, date, and the notation "Inactivated, unabsorbed."

The serum will remain in good condition for a longer period if left unabsorbed. Absorb only enough for several months' use.

Absorption of anti-M serum:

1. Using all sterile precautions, dilute the serum 1:20 with sterile saline.
2. Place 10 ml. diluted serum in one tube and 10 ml. in another.
3. Place 5 ml. of washed, packed, pooled group ON, AN, and BN cells in each anti-**M** diluted serum.
4. Cork the tubes and mix by inverting back and forth about 6 times; then allow to stand for 30 min.
5. Centrifuge and test for the presence of anti-**M** or anti-**N** agglutinins as described above. Carry out the absorption at room temperature. Absorption is complete in the case of anti-**M** fluids when there is no longer a reaction with type N bloods, while the serum still agglutinates types M and MN cells within a 1 or 2 min. period.

Absorption of anti-N serum: Great care must be exercised not to overabsorb the serum. Carry out the absorption slowly to approach the end point of complete absorption very accurately.

1. Use groups OM, AM, and BM pooled, washed, packed cells to absorb the anti-**N** serum, adding the cells to the diluted serum as with anti-**M** absorption.
2. Keep the mixture of anti-**N** fluid and type M cells in a glass of warm water at 37° C. in an incubator for 30 min.
3. Then remove from the incubator and test. If the antiserum has not been completely absorbed, add more cells, using only half the above quantity, and repeat the same technic in the incubator.

This serum must agglutinate types N and MN cells within 1 or 2 min. but must not agglutinate type M cells within a 10-20 min. period.

Assuming that the M cells are not agglutinated by the anti-**N** serum and the N cells are not agglutinated by the anti-**M** serum, but each antiserum clumps its specific cells, the 2 products may be bottled. Some laboratories add a small amount of Merthiolate, 1 part of 1% solution to 100 parts of testing fluid to give a 1:10,000 concentration, to prevent growth of bacteria.

Levine[234] recommended toluol as a preservative for anti-**M** and anti-**N** agglutinins in testing fluids. Add 1 drop of toluol to each ml. of diagnostic fluid. Stopper and seal with paraffin. After 32 mo. Levine found that the anti-**M** was just as active as when freshly prepared. The best way to preserve antisera is in the frozen state without the addition of any chemical.

In working with anti-**M** and anti-**N** testing fluids, as shown by Landsteiner and Levine, bloods containing factor **N** alone are more strongly agglutinated by anti-**N** testing fluids than bloods containing **M** and **N** together. Type N blood is about twice as sensitive as type MN blood to agglutination with anti-**N** serum.[233] It must be emphasized that control bloods of all 3 types are necessary in the performance of M and N tests. Wiener emphasized that, in a large experience, using 3 different anti-**M** fluids and 3 different anti-**N** fluids, he had never found a discrepancy among the reactions. However, with the recent discovery of the M^e factor, this is no longer true (p. 1551).

There are certain sources of error in the preparation of these antisera, which Wiener has outlined. First, great care must be used in the preparation of anti-**M** and anti-**N** testing fluids. Overabsorption is the fault of most beginners in making these fluids. Wiener has shown that there may be interfering reactions caused by specific agglutinins other than those directed against M and N. Similar interfering reactions may be caused by natural or immune agglutinins against A and B, and there may be coexistence in the same serum of more than one of the agglutinins against M and N. To obviate these difficulties, the preparation of the testing fluid by absorption should be carried out by using a mixture of bloods of types OM, AM, and BM. Again Wiener has shown that there is danger of false positive reactions from underabsorption of the immune sera. This particularly applies to the tests for **N** but may be prevented easily by the inclusion of negative control bloods.

LUTHERAN BLOOD SYSTEM

Blood group factor Lu

Callender et al.[235] reported an antibody, later described in more detail by Callender and Race in 1946,[236] found in the serum of a patient with disseminated lupus erythematosus; this patient had received a number of blood transfusions and produced a number of isoimmune antibodies. The name of the donor was Lutheran, and the antibody was designated anti-**Lutheran.** The antigen, at first called **Lutheran,** could not be given the designation "L" because of possible conflict with the **Lewis** antigen. The term **Lu** was later used to designate this antigen. Later the notations $\mathbf{Lu^a}$ and $\mathbf{Lu^b}$ were agreed upon for the antigen and its reciprocally related factor. The phenotypes are described as Lu(a+) and Lu(a–). The corresponding antibodies are anti-$\mathbf{Lu^a}$ and anti-$\mathbf{Lu^b}$. Anti-$\mathbf{Lu^b}$ was discovered by Cutbush and Chanarin[237] in 1956 and by Greenwalt and Sasaki[238] in 1957. $\mathbf{Lu^a}$ was shown by Callender and Race to be inherited as a mendelian dominant.

The incidence of Lu(a+) was found to be 6.93%. Wiener[214] states that if heredity by a pair of allelic genes Lu^a and Lu^b is postulated, the genotype frequencies calculated from these data are Lu^aLu^a, 0.12%; Lu^aLu^b, 6.81%; and Lu^bLu^b, 93.07%.

The antibodies for $\mathbf{Lu^b}$ have been found after isoimmunization during pregnancy and as a result of a blood transfusion.

J-j SYSTEM—KIDD BLOOD GROUPS (ALSO CALLED Jk^a AND Jk^b)

In 1951 Allen et al.[239] described an antibody, in the serum of a mother of an erythroblastotic baby, which was found to be serologically independent of the other blood systems. The patient in whom the antibody was found was named Kidd, which gave the designation **Kidd** to the antigen identified by the new antibody. Since the Kell-Celano system had already utilized the K-k symbols, the designation Kidd was later changed to $\mathbf{Jk^a}$, and when the reciprocal factor was found, it was called $\mathbf{Jk^b}$. These terms were later changed to **J** for $\mathbf{Jk^a}$ and **j** for $\mathbf{Jk^b}$.

The reciprocal antibody, anti-$\mathbf{Jk^b}$, was found in 1953 by Plaut et al.[240]

The incidence of factor **J** is approximately 75-77% among Caucasoids; among Negroids it is 90%, and among Mongoloids it ranges from 50-100%.

Identification of factors **J** and **j** are made by the use of anti-**J** sera, using the antiglobulin technic, since other tests usually fail to detect the antigen. The tests for these factors, therefore, have the same limitations as do the Duffy tests. Moreover, the antisera tend to lose their activity upon storage so that the tests must be repeated several times, using Wiener's blind technic, and including controls of known types J and j. Anti-**j** serum is not as yet easily available.

Testing for J factor

1. Wash the unknown cells 3 times in saline and resuspend to a 2 or 3% suspension. These cells are **not** to be enzyme-treated.
2. Place 1 drop of the cell suspension in a small tube (7 mm. inside diameter) and add 1 drop of anti-**J** serum.
3. Prepare positive and negative control cells and treat in the same manner.
4. Place in a water bath at 37° C. for 1 hr.
5. Read the sediment macroscopically and under a scanning lens.
6. If the reactions are negative (as they probably will be since the **J-j** factors require antiglobulin tests for their detection), wash 3 times in saline and remove all traces of saline after the last washing. Turn the tubes upside down and strike the lip hard against a gauze sponge after the last washing.
7. Add 1 drop of antiglobulin serum (Coombs), mix, and centrifuge at 500-1000 r.p.m. for 1 min.
8. Gently dislodge the sediment and read as before.

If the positive and negative controls give correct readings, then agglutination means the presence of factor **J** and lack of agglutination indicates its absence.

DIEGO BLOOD SYSTEM—FACTOR Di

Levine et al.[241] found an antibody in the serum of a mother of an erythroblastotic baby, which identified blood factors serologically distinguishable from those of other known blood group systems. The family name was Diego, and the antigen identified by the antibody became known as the **Diego** or **Di** factor. It seems to be common among South American Indians but was not found in tests on 2600 consecutive Caucasoid blood specimens.[242] Layrisse and Arends,[243] in a series of tests, found the **Di** factor to be common among Chinese, Japanese, pure-blood Negroes, North American Indians (incidence 2-45%), Eskimos, and Polynesians. The inheritance of the **Di** factor has been confirmed as a simple mendelian dominant. It has been found in 4 generations of a single family.

OTHER BLOOD GROUP FACTORS—HIGH- AND LOW-FREQUENCY FACTORS

Some authors use the term "public antigens" to refer to high-frequency factors, those possessed by the vast majority of people. Since we prefer the term "high-frequency" factors, it will be used here.

Some high-frequency factors are blood factor **H** of the A-B-O system, **U** of the M-N-S-U system, **hr″** of the Rh-Hr system, **Tj**^{a} of the P system, and factors **k** and **Kp**^{b} of the K-k (Kell-Cellano) system. Four other high-frequency factors are **Vel**, **Yt**^{a}, **I**, and **Sm**.

High-frequency blood factors

(1) **Blood factor Vel:** In 1952 Sussman and Miller[244] tested the serum of a patient who had had a transfusion reaction, against 10,000 samples of group O blood, and found that all but 4 of these were agglutinated by the serum. Since the patient's name was Vel, the antibody in the blood was called anti-**Vel**. Levine and associates reported a second example of anti-**Vel** and showed it to be different from anti-**Tj**^{a}, anti-**U**, and anti-**H**.

The Vel factor has a frequency of 99.96%, so that negatively reacting bloods are extremely rare. No naturally occurring anti-**Vel** antibodies have been reported, and isoimmune antibodies have been found only rarely.

The anti-**Vel** of Sussman and Miller clumped incompatible red cells suspended in saline but with a titer of only 4 units, although the titer was 32 units in plasma medium. The patient gave a history of previous pregnancies and transfusions, to which her isosensitization could be ascribed. Sussman and Miller carried out tests on relatives of the patient and on those of the 4 **Vel**-negative donors whom they found, but each of the 8 siblings proved to be **Vel** positive. On the plausible assumption that **Vel**-negative individuals are homozygous, genotype *vv* for a rare gene, the frequency of this gene *(v)* was calculated to be approximately 2% and that of its allele *(V)*, 98%.

A second case of sensitization to the **Vel** factor was reported in 1955 by Levine et al.[245] This was a woman who had had previous pregnancies and transfusions which could have sensitized her. Cross-matching tests carried out preliminary to another transfusion proved every one of 1000 successive random donors incompatible. Unlike the original serum of Sussman and Miller, however, the freshly drawn serum of the second patient caused hemolysis of all red cells tested, whereas the patient's inactivated serum caused agglutination. The patient's red cells were tested with the original anti-**Vel** serum and proved to be Vel negative, and the antibody in her serum was shown to correspond to anti-**Vel**.

A third case of **Vel** sensitization was reported in 1959 by Bradish and Shields[246] in a male patient who had been sensitized by transfusions, and the serum reacted like that of the patient described by Levine. Higher titers were obtained in plasma medium than in saline medium, and the serum also reacted by the indirect antiglobulin technic.

Recently (1961) Levine et al.[247] described another sensitized **Vel**-negative individual who had had a hemolytic transfusion. Her antibody produced both hemolysis and agglutination. Family studies disclosed 7 Vel-negative persons in 3 generations.

Strickel et al.[248] described 2 sisters who both lacked the **Vel** factor. Wiener et al. in 1961[249] described still another case of **Vel** sensitization discovered in the course of routine cross-matching tests preliminary to blood transfusion. Tests were also carried out on relatives of the patient in a search for compatible donors. The patient was group O MN k Rh_2rh. Every group O Rh-positive blood was incompatible with her serum, which hemolyzed the cells, although hemolysis was only about 5-50%. In titrations, hemolysis was observed in saline dilutions of her serum up to 1:4 but not in higher dilution. The antiglobulin test was made after washing the unlysed cells in saline 3 times; clumping occurred. The antibody titer by the antiglobulin technic was 30 units. Ficinated cells were also lysed by the patient's unheated serum, and this was more pronounced than with the unmodified cells. Her serum did not hemolyze or agglutinate her own red blood cells. The direct antiglobulin test was negative. She was not autosensitized.

Tests with 200 donors yielded no compatible bloods. She was type k and **hr″** positive, which ruled out possibility of sensitization to these 2 factors. The behavior of the antibody did not correspond to that which is characteristic of **hr″** or **Kell** antibodies. She was U and **I** positive as well as **H** positive. By using anti-**Vel** serum it was shown that she was **Vel** negative. Laboratory tests showed that this patient had no autosensitization.

The problem of transfusing a sensitized **Vel**-negative individual is very difficult to resolve. The simplest answer is prevention of sensitization. Since **Vel**-negative persons are possibly or probably homozygous for a rare recessive gene, whenever such a person is found all siblings should be tested. If any should prove to be **Vel** negative, he should be so informed and given a suitable identification card warning that no transfusions be given except in dire emergency. If transfusion is essential and time permits, one of his siblings known to be **Vel** negative and compatible should be selected as a donor in order to avoid sensitization. Once sensitization has occurred, obviously no transfusion is entirely safe unless a **Vel**-negative donor is used. Where there are no **Vel**-negative siblings, a central clearinghouse at which the names and addresses of donors of rare blood types are kept on file may supply the blood. The central clearinghouse of the American Association of Blood Banks lists 12 **Vel**-negative donors.

When no **Vel**-negative blood is available, Wiener and associates suggest that the patient may be a donor for himself. For example, in the case of cardiac or other elective surgery which may be scheduled months in advance, the patient can be bled periodically and his blood frozen or stored in ACD solution in a refrigerator, depending on the circumstances; then when the patient is subjected to surgery, his own blood can be used as needed.

(2) **Blood factor Yt^a:** In 1956 Eaton et al.[250] described an antibody, anti-**Yt^a**, which reacted with the great majority of blood cells tested. The patient had had several transfusions before the presence of anti-**Yt^a** was found in an incompatible cross matching. It reacted with a modification of the indirect antiglobulin test and with the trypsin antiglobulin test. It was found to be unrelated to the other known blood group systems. Another example of isosensitization to **Yt^a** was later found.[181] It must be pointed out that only those who lack the factor can form the antibodies, and the scarcity of isosensitization shows the almost universal nature of the factor **Yt^a**.

(3) **Blood factor I:** In 1956 Wiener et al.[251] reported their finding of an isoantibody of high titer in the serum of a group A_1B woman with acquired hemolytic anemia, which proved to be of the cold antibody type. Her serum also showed another isoantibody, reactive at room temperature with almost all other human blood cells but not with the patient's own cells. The factor detected by this patient's serum proved to be different from all other previously found blood factors. Because of the high degree of individuality of blood specimens which failed to react with the patient's antibody at room temperature, the antibody was designated anti-**I**, and the antigen it identified was called **I**. Further examples of the same antibody have been reported in 3 environments[252]: (a) anti-**I** in the serum of patients with acquired hemolytic anemia of the cold antibody type, of which there were 9 examples; (b) anti-**I** in sera which in the past had been said to contain a "nonspecific complete cold autoagglutinin," of which there are more than 50 reported incidents[253]; and (c) anti-**I** in the sera of normal persons whose red cells have abnormally little **I** antigen. There are 6 examples of this. Shapiro found a case of anti-**I** in South Africa.[254]

Wiener designated those persons whose blood contained the factor as I and those who lacked it as i (**I** negative). Among a series of 22,964 random donors, only 5 proved to be **I** negative, and 4 of these were Negroids. The **I** antigen is very feebly developed at birth.

Variants of the I agglutinogen have been suggested because of the markedly different titers which were obtained against different cells, ranging from 25 to 5000 units—I_1, I_2, I_3, etc., and i.

W. Weiner et al.[255] demonstrated in 1959 that 2 of their patients with acquired hemolytic anemia of the cold antibody type had the antigen **I** on their red cells and anti-**I** in their serum. The autoantibodies of patients with acquired hemolytic anemia of the warm antibody type can often be shown to be specific for known blood group antigens possessed by the patients. It has now been shown that some patients with acquired hemolytic anemia of the cold antibody type have the **I** antigen on their cells and anti-**I** in their serum.

(4) **Blood factor Sm:** Schmidt et al.[255a] reported a new antibody, anti-**Sm**, in the serum of a mother apparently immunized by pregnancy.

Low-frequency blood factors

Low-frequency blood factors have been called "private" blood factors by some investigators since they seem to occur only in particular families. A number of such low-frequency antigens have been found: **Mi** and **Gr** of the M-N-S-U system, **rh**^{w}, **rh**^{w2} and **rh**^{x} of the Rh-Hr system, and **Kp**^{a} of the K-k system. In addition to these, there are the **Leavy, Jobbins, Becker, Ven, Be**^{a}**, Ca,*** **Rm,** and **By** factors. Most of these have been found in hemolytic disease, some were stimulated by pregnancy, and some were stimulated by transfusion. These antigens are distinguishable by specific antisera and are independent of the 9 blood group systems.

With all the factors, positively reacting bloods are very rare. No naturally occurring isoantibodies have been reported to date, and isoimmune antibodies are very rare.

Factor Ca

Wiener and Brancato[256] described the results of their studies on a rare hereditable human agglutinogen discovered during the study of a puzzling case of erythroblastosis fetalis. This baby and her mother were both Rh negative, and no A-B-O blood group incompatibility could be demonstrated. The problem of specificity of the antibody remained unsolved for 8 yr. until newer and more sensitive methods for detecting antibodies were devised, the antiglobulin and enzyme technics. At that time it was possible to demonstrate in the mother's serum, in addition to an **Rh**$_{o}$ antibody, an antibody for a second blood factor present in the child's blood cells. The factor was given the designation **Ca** from the family name (Cavaliere). This factor was not found in blood cells of 48 consecutive type rh bloods taken at random but was present in 4 out of 7 type rh individuals in the patient's own family. No **Rh**$_{o}$ variant was demonstrable in the patient's cells.

The **Ca** factor seems to be a rare hereditable blood factor probably independent of Rh-Hr types.

Factor Xg and a sex-linked blood group

In 1962 Mann et al.[257] described the first sex-linked blood group antigen, designating it as **Xg**^{a}, the new antibody as anti-**Xg**^{a}, the phenotypes as Xg(a+) and Xg(a–), the gene responsible for the antigen as Xg^{a}, and the gene for its so far silent allele as Xg. Random tests of 342 Caucasians showed the following distribution:

Of the females 88.83% were positive, and of the males 61-69% were positive. The difference is overwhelmingly significant ($\chi^2 = 34.8$ for 1 d.f.).

Xg(a+) males are of the genotype $Xg^{a}Y$, and Xg(a–) males are of the genotype $Xg\ Y$. Xg(a–) females are of the genotype $Xg\ Xg$.

The authors compared the expected distribution of the Xg groups in Caucasian parents and offspring with the results actually observed in testing 50 Caucasian families with 104 children. Details of the families illustrate very clearly certain rules which may be laid down for an X-borne dominant antigen:

1. From the mating positive × positive, there can be no negative daughters; only sons may be negative, which is the most striking feature in this situation.
2. From the mating positive male × negative female, all sons have to be negative and all daughters positive, with which the present findings conform.
3. From the mating negative male × positive female, all sorts of children are possible, and all sorts have been found.

Other rules, corollary to these, are as follows: of positive men, the mothers and daughters must be positive; of negative women, the fathers and sons must be negative. To these rules the families also conform, as do the members of 25 families lacking one parent. These rules apply to ordinary males and females and need not necessarily apply to people with Turner's syndrome (XO) or with Klinefelter's syndrome (XXY).

There is little reason to hope that another example of the antibody will be found for years, because innumerable Xg(a–) persons must have been transfused with Xg(a+) blood; yet until now no one is known to have produced the antibody. This statement has been amended by Mann et al. (see below).

***Wr**^{a} is omitted from this list because it has been shown to be the same as Wiener and Brancato's earlier described **Ca** factor.

A second example of anti-**Xg**^{a} has been reported (1963) by Cook et al.,[257a] which, like the first, could be detected only by the antiglobulin test, and this antiglobulin serum must contain either an adequate amount of anti-β_1 (complement) globulin or an exceptionally large amount of anti-7S gamma globulin. As tests for the antibody improve there is little doubt that other examples will be found.

The antigen is well developed at birth, 8 out of 12 cord samples having been found to be Xg(a+); and of 4 fetal samples 2 were Xg(a+) and 2 were Xg(a–).

The number of Negroes so far tested are few, but the studies made of these few suggest that the distribution of the antigen is about the same as that in Caucasians. Tests on 61 New York and West Indian Negroes showed 26 males and 15 females as Xg(a+) and 18 males and 2 females as Xg(a–).

Mann and associates believe that the antigen may play a leading role in helping to plot the relative position of genes on the X chromosome. In collaboration with many other experts, they are beginning to test families with red-green color blindness, hemophilia, Duchenne's type of muscular dystrophy, glucose-6-phosphate dehydrogenase deficiency (G-6-PD), and other, rarer characters known to be controlled by X-borne genes. If the Xg locus is close to the hemophilia or to the Duchenne locus, then in some families it would be possible to distinguish normal women from carriers with an error in proportion to the recombination frequency. The Xg groups should be of aid in mapping the X chromosome and in problems of dosage compensation; they should be useful in certain families in attributing to oögenesis or to spermatogenesis the nondisjunction which is responsible for Turner's and Klinefelter's syndromes.

Chapter 79

Whole blood transfusions*

ADDINE G. ERSKINE

The transfusion of whole blood is accompanied by certain risks, such as unintentional transfusion of incompatible blood due to clerical errors, incorrect identification of the recipient (laboratory errors), as well as transmission of certain diseases, notably hepatitis (p. 1568). Transfusions should be undertaken only when clear-cut and substantial indications exist. Mild hemoglobin deficiencies can usually be corrected by satisfactory dietary intake and supplementary medication. Whole blood transfusions should be reserved for patients who are refractory to such treatment or in situations requiring immediate replacement of blood. Even emergency blood transfusions should never be attempted without proper laboratory preparations since plasma may be given without blood grouping tests and an osmotic pressure almost equal to that of whole blood can be obtained by the use of plasma or albumin.

Whole blood should be available at all times to prevent any delay necessitated by a search for donors. Although this compromises the freshness of the blood, the use of stored blood is essential (refer to blood banks, p. 1582). The blood requirements of most patients can usually be met by blood stored up to 21 days, but certain conditions require transfusion of fresh blood (less than 24 hr. old).

INDICATIONS FOR WHOLE BLOOD TRANSFUSIONS

Hemorrhage: The principal indication for transfusion of whole blood is sudden loss of blood from hemorrhage. Such cases call for immediate transfusion of whole blood. In addition to this acute blood loss, loss of blood in hemorrhagic diatheses requires replacement with whole blood. Two significant factors enter into the body's defense against hemorrhage[258]: (1) coagulability of the blood and (2) vascular response. Defective coagulating mechanisms are seen in hypoprothrombinemia, thrombocytopenia, hemophilia, and fibrinogenopenia. Plasma is used in preference to whole blood in peripheral circulatory failure or shock.

Surgery: The preoperative preparation and postoperative care of patients may require transfusions of whole blood when there is impaired nutrition, secondary anemia, or lowered resistance. In hypoproteinemias plasma is preferred over whole blood, or a better procedure is an initial injection of plasma followed by the injection of whole blood.

Anemias: In addition to the hemorrhagic states, transfusion of whole blood is necessary in anemias of blood loss or those with increased erythrocyte destruction of hereditary or acquired nature. The basic principle of blood transfusion utility in anemia is a replacement therapeutic procedure. The usefulness of transfusions in anemias due to increased destruction or excessive hemolysis is seen in the good results in familial hemolytic jaundice, sickle cell anemia, erythroblastosis, and many acute and chronic types, as well as in Cooley's anemia and others.

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*We wish to express our thanks to the American Association of Blood Banks, Chicago, Ill., for permission to use parts of Chapter VI of their Technical methods and procedures, revised edition, 1960, in the preparation of this chapter.

Leukemias: Transfusions may be indicated in leukemia. No particularly brilliant results are seen in the chronic leukemias except, perhaps, relief of the associated anemia.

Leukopenia: In leukopenia the rationale for transfusion is only of a supportive nature. According to Doan,[259] transfusion is proper in neutropenic states because normal blood contains nucleotides which stimulate the formation of these neutrophilic cells. Transfusions have been used in agranulocytosis with varying success in different hands.

Infections and poisonings: Blood transfusions are used in many acute and chronic infections and in other intoxications and poisonings. Plasma injections, however, have been even more useful.

CONTRAINDICATIONS FOR BLOOD TRANSFUSIONS

Contraindications for whole blood transfusions are principally acute pulmonary edema, cardiac decompensation, and massive pulmonary embolism or infarction.

HEPARIN AND TRANSFUSION

The Scandinavians have been using heparinized blood in transfusions for a number of years. It eliminates the disadvantages of sodium citrate as an anticoagulant and greatly simplifies the procedure of transfusion.

REQUIREMENTS FOR FRESHLY DRAWN BLOOD

Bleeding due to disorders in the clotting mechanism

Hemophilia: Since the antihemophilic factor of plasma is labile, blood for hemophiliacs should be as fresh as possible. Fresh frozen plasma, antihemophilic globulin, or dried plasma prepared from fresh blood may be used.

Afibrinogenemia: In many cases administration of commercially available purified fibrinogen is preferable to whole blood.

Hypoprothrombinemia: Prothrombin is also labile. Hypoprothrombinemia is usually present in jaundiced patients and in patients under Dicumarol therapy. (Some authorities believe that bank blood retains a high level of prothrombin for at least 1 wk.)

Thrombocytopenic purpura: Platelets, the corrective factor of blood in thrombocytopenic purpura, are labile and are most viable within 4 hr. after withdrawal of the blood. Some of the lability of the platelets may perhaps be overcome by the use of plastic bags or siliconed bottles for blood withdrawal, by the use of plastic tubing, and by coating with nonwetting agents those surfaces that come in contact with the blood, although the age of the blood is more important than the container. In some cases, however, substances supplied by the platelets are all that are necessary, and viable intact platelets are not needed.

Hypoplastic anemias

The longer the transfused red cells remain alive in the patient's blood stream following transfusions in patients with hypoplastic anemia, the more benefit such patients derive from transfusions. This is especially true of those patients who require large amounts of blood.

Erythroblastosis fetalis

In erythroblastosis fetalis the need is for long-lasting compatible red blood cells. The liver of a newborn infant is often unable to excrete the pigment resulting from breakdown of old transfused cells, and use of stored blood may make the pre-existing jaundice worse. In addition, for a period of several weeks the baby's bone marrow may fail to produce enough red cells to keep pace with the normal rate of destruction so that the transfused cells must carry the baby through this period.

Erythroblastosis fetalis due to Rh incompatibility: Opinions differ on the A-B-O blood group that should be given to erythroblastotic babies of groups A, B, or AB. Some use only low titer group O blood to which 1 or 2 vials of group A and group B group-specific substances are added. Table 343[260] shows how to select the proper blood group[261] if group O blood is not to be used routinely. Still others use group O blood after sedimentation of the red cells and removal of the plasma. Some use group O cells to which AB plasma has been added. Bank blood, regardless of its age, is better than no blood at all. Unger uses group O blood from which the plasma has been removed. He **never** uses group A and group B substances. In his opinion the only alternative is to use blood compatible with the mother's blood. Even if group O blood is used, it should be cross matched with the mother's blood and the major cross match **must** be compatible.

The Rh type of the blood used, usually Rh negative, must be compatible with the ma-

Table 343. *Selection of proper donor blood in exchange transfusions in Rh-Hr erythroblastosis**

Infant's group	*Mother's group*	*First choice of donor group*
A	A or AB	A
B	B or AB	B
AB	B	B
AB	A	A
AB	AB	AB

*From Wiener, A. S., Samwick, A. A., Morrison, H., and Cohen, L.: Exper. Med. & Surg. 11:276, 1953.

In all other instances use low titer group O blood or group O blood from which the plasma has been removed. By removing the plasma one knows that the titer of anti-**A** and anti-**B** is low.

ternal antibody as proved by suitable cross-matching tests. Always use Rh-negative blood if anti-**Rh**$_0$ is the cause of the erythroblastosis.

In an emergency, when the proper blood for the baby is not available, the mother's blood may be safely used despite its antibody content, according to many authorities, but if time and facilities are available, it is safer to remove the plasma and perhaps replace it with AB plasma, or wash her cells.

Erythroblastosis fetalis due to A-B-O incompatibility: The baby should receive low titer group O blood of the same Rh type as his own, i.e., Rh negative or Rh positive, or remove the plasma or wash the donor's cells.

Erythroblastosis fetalis due to an unidentified incompatibility: In these rare instances it is safe to use blood if the donor's cells are compatible with the mother's blood. The matching tests must include the antiglobulin method, and ficinated cell tests should also be made.

SMALL UNITS OF BLOOD

Small units of blood may be prepared by taking blood from the selected donor into several small containers at the time of donation. There are available small commercial evacuated bottles containing 15, 40, and 60 ml. ACD solution. The bottles are graduated so that the proper quantity of blood may be introduced into each bottle.

A Y-shaped plastic donor set suitable for drawing blood into several bottles is commercially available. Blood runs through the donor needle into a short length of plastic tubing; it then reaches a bifurcation where the tubing splits in two. On each end of the 2 lengths is a bottle needle. Each needle is inserted into a separate bottle. One bottle is filled while the tubing leading to the second is clamped off. When the first bottle is filled, its tubing is clamped shut and the second tubing unclamped. The first needle is then inserted into an empty bottle and so on, until the phlebotomy has been completed.

Some blood banks make a practice of keeping several small units of low titer group O Rh-negative blood for pediatric use.

EXTRACORPOREAL CIRCULATION

The increasing use of procedures involving circulation outside the body (extracorporeal circulation) during open-heart surgery has introduced new problems to the blood bank.

The following are points of difference from customary blood bank routine.

1. To ensure freshness, donations are made either on the afternoon before or the morning of contemplated use. Bloods drawn the day before are refrigerated.

2. To minimize hemolysis, donations are made (a) using the gravity method or (b) using nonwettable surfaces for collection and storage.

3. Blood is collected in a heparin solution in saline and used to prime the machine. The balance is collected in ACD solution. Since the heparin solution prevents clotting for only 24 hr., the blood must be used within this period or be discarded, unless it is collected in a double plastic bag, one with heparin and one with ACD; then the heparinized blood is transferred to the plastic bag with ACD, after which it has the usual 21-day expiration date.

4. To minimize other possible complications, (a) donors must fast for at least 4 hr. prior to donation, (b) blood must be mixed thoroughly during collection to prevent coagulation, and (c) blood grouping and cross-matching tests must be performed with meticulous care.

PACKED RED BLOOD CELLS

Avoid contamination in preparing packed cells.

Preparation of packed cells from blood collected in a bottle:

1. Collect the blood in a standard ACD anticoagulant solution.
2. Keep the blood refrigerated while the cells sediment or centrifuge the blood at 2000 r.p.m. for 60 min. after it has remained refrigerated for 24 hr.
3. Then remove the plasma within 48 hr. after collection. To do this, disinfect the rubber stopper in the blood bottle with tincture of iodine or other acceptable disinfecting solution.
4. Perforate the large free hole in the rubber

stopper using the needle attached to a sterile air filter; then insert a long plasma aspirating needle, taking care to keep the needle **above** the plasma surface. Insert the needle of the air filter through the **X mark** in the rubber stopper and not through the small free hole leading to the air vent tube.

5. Remove the supernatant plasma aseptically, aspirating it into a sterile evacuated bottle. Immediately remove the air filter to seal the bottle containing the cell sediment.

If the cells have not been centrifuged, they may be transfused without addition of resuspending fluid, especially if the cells are well shaken before administration and a needle no smaller than 18 gauge is used in the recipient set. If a resuspending fluid is desired, ½ vol. or more of physiologic saline is added to the packed cells just prior to their administration. If the blood is to be centrifuged, cross matching is carried out beforehand, since the pilot tubes must be removed from the bottle in order to centrifuge it.

Packed or resuspended red blood cells should not be accepted for credit if returned to the blood bank, and in no case may they be reissued to another patient.

If packed cells are prepared this way, they **must** be used within 24 hr. of the time of preparation, although preferably sooner.

Preparation of packed cells from blood collected in a plastic bag: Commercially available plastic bags are adaptable to the preparation of packed cells. The bag may be centrifuged if desired; an air filter is not necessary; a length of tubing is attached between the blood bag and another container; the plasma is "squeezed" out of the bag by pressure on its surface. The remaining packed cells may then be administered from the original container. Since this is a closed system, the date of expiration of the cells is the same as that of the blood from which they were prepared.

CHOICE OF DONORS

The choice of donors is very important. The proper blood group should be selected as determined by the tests described. In addition, the donor must be in good physical health and able to withstand the withdrawal of 500 ml. blood without injury to health. (Refer to the section on Red Cross requirements.)

Universal donors

The term universal donor is applied to all persons of group O because, although their sera contain anti-**A**, anti-**B**, and anti-**C** agglutinins, their cells are not agglutinable by anti-**A** agglutinin (present in group B serum) or by anti-**B** agglutinin (present in group A serum). Since group O cells contain neither A nor B agglutinogen, it was previously believed that transfusion of group O blood into any individual would result in a compatible transfusion. This has proved to be incorrect. If the antibody titer of group O serum is high and group O whole blood is transfused into an individual of group A, B, or AB, a reaction may take place. Such blood should be used only in a genuine emergency.

Group O serum often contains isohemolysins in addition to isoagglutinins, and these are even more dangerous than the isoagglutinins.

In order to use group O donors for patients in group A, B, or AB, the anti-**A** and anti-**B** titer of the donor's serum must be ascertained by titration (p. 1476), and only those group O donors whose antibody titer is low (below average) should be used. Better still is a quicker procedure advised by Unger, namely, remove the plasma. It must be stressed that the use of the universal donor principle is not recommended and in some cases has led to disastrous results.

Witebsky substance, or A-like and B-like blood group specific substance

The first study on isolation of an A-like blood group-specific substance was carried out by Brahn and Schiff,[262] Freudenberg et al.,[263] and Landsteiner,[264] who had first isolated an A specific carbohydrate-like substance from horse saliva in 1932. Others have determined the chemical nature of the substances. An A-like blood group-specific substance occurs in relatively large amounts in commercial preparations of pepsin and peptone. Witebsky and Klendshoj[265, 266] isolated a B-like blood-specific substance from the stomach juice of group B subjects, using a method similar to that in which Goebel[267] isolated the A-like substance from commercial peptone. The B-like substance proved to be carbohydrate-like and was as potent as the carbohydrate-like A substance.

The presence of anti-**A** and anti-**B** isoagglutinins is at times ignored in the so-called universal group O blood plasma because of the dilution to which the serum of the donor is subjected in blood transfusions. When 500 ml. blood are given to adults in 1 blood transfusion, the resulting dilution is about 1:10. It is obvious, therefore, that blood plasma with high titers might be detrimental if introduced into the bloodstream of a patient of group A, B, or AB, particularly, according to Unger, if the patient has had a hemorrhage or is a child. It is, of course, likely that group substances in the blood as well as in the tissues capable of combining with isoantibodies represent a factor which, in addition to the dilution factor, is responsible for the fact that the isoantibodies in group O blood do not produce more damage than has been reported when group O is used as a universal donor, as noted by Wiener.[268]

Note: Unger calls attention to the fact that group O blood contains both bivalent and univalent antibodies, so that when titrations are made one must be sure to determine the titer of the univalent antibodies as well as of the bivalent antibodies. The univalent antibodies are not neutralized by group A and group B substances and are dangerous.

Witebsky found that the group A-like specific substance markedly inhibited the agglutination of human blood cells of group A by serum of group B. At a dilution of 1:250 his stock solution completely inhibited agglutination under his experimental conditions, whereas some inhibitory influence was detectable even in dilutions of 1 in several millions. Moreover, this substance was markedly heat stable when dissolved in saline. He also showed the lack of toxicity of this substance in work on guinea pigs. No agglutination of human red blood cells of group A by plasma of group O treated with group-specific substance occurred when the dilution of the plasma was as much as 1:256 or more. It is now believed that addition of the A-like blood group-specific substance to group O blood is effective in reducing the titer of the natural bivalent anti-**A** agglutinin in group O blood to an extent that renders this agglutinin practically ineffective. It must again be pointed out that this is true only of the bivalent and **not** the univalent antibody. It is **not** harmless and may stimulate immune antibodies in a female and be responsible for subsequent erythroblastosis.

Witebsky et al.[269] gave specific directions for preparation and transfusion of universal group O blood to patients outside the group. The Sanitary Code of the State of New York was amended and the amendment adopted by the Public Health Council on June 26, 1940, requiring that group O blood be titrated before it is used as universal blood and that no group O blood with high titers be permitted to be used for patients of other blood groups. Gesse[270] described 46 cases of hemolytic shock following transfusion of universal blood, 20 of which ended fatally.

The use of universal donor blood should be discontinued except in a real emergency when homologous blood is not available.

Blood group-specific substances A and B are commercially available.* They are supplied in 10 ml. sterile vials. The contents of 1 vial (10 ml.) are added to 500 ml. of group O blood and mixed **just prior** to use in transfusions. Because of the danger of stimulating increased antibody formation in female patients potentially capable of childbearing, use of group O blood modified by group-specific substances should not be permitted except in the face of an emergency. Instead of using group O blood treated with group-specific substances, remove the plasma and use the remaining cells, meanwhile carrying out proper blood grouping and cross-matching tests. To render this even safer, the donor blood should also be Rh negative. Tisdall et al.[271, 272] concluded with respect to this entire matter as follows:

1. As little as 25 ml. of group B plasma containing a high titer of isoagglutinins may cause a hemolytic reaction when administered to a group A recipient.
2. A-like and B-like group-specific substances will reduce a high isoagglutinin titer of bivalent antibodies to low levels. Unger points out that it will in no way affect the titer of univalent anti-**A** and anti-**B**, and therein lies one of the dangers of using such substances.
3. A and B specific substances are capable of antibody stimulation even when in combination with anti-**A** and anti-**B** agglutinins of group O blood. This calls for some caution in administering such material to women potentially capable of childbearing.
4. For use as universal donor blood in civilian hospitals, Rh-negative group O blood, selected for its low natural isoagglutinin titer, is preferable to random group O blood to which group substances have been added to reduce the isoagglutinin titer. The plasma should be removed before administering the blood.

Goebel technic for preparing group-specific substance A[267]

1. Dissolve 450 gm. peptone (Difco Neopeptone) and 180 gm. sodium acetate ($3H_2O$) in 1350 ml. distilled water.
2. Precipitate the crude fraction containing the A substance by adding 3375 ml. 95% alcohol.
3. Collect the precipitate in centrifuge tubes the following day and redissolve in 270 ml. distilled water.
4. Discard the undissolved sediment after centrifugation.
5. Collect the insoluble fraction again by adding

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*Sharp & Dohme, Philadelphia, Pa.

45 gm. sodium acetate and 675 ml. alcohol.

6. Repeat a third time, using 150 ml. distilled water and 24 gm. sodium acetate, and precipitate with 2.5 vol. alcohol.
7. Redissolve the precipitate in 180 ml. water.
8. Add 27 gm. sodium acetate.
9. Adjust the reaction to pH 4.8 by adding 20% acetic acid.
10. Deproteinize by shaking for 16 hr. with a mixture of 40 ml. chloroform and 8 ml. butyl alcohol.
11. The resulting solution should give negative tests for protein with 20% trichloracetic acid, 5% sulfosalicylic acid, and saturation with picric acid.
12. Recover the fraction containing the A substance by precipitating with 2.5 vol. 95% alcohol.
13. Dialyze a solution of this substance in water through cellophane against successive changes of distilled water. Keep refrigerated during dialysis.
14. Precipitate the active fraction in 10 vol. acetone.
15. The substance tends to remain in colloidal suspension but forms a flocculent precipitate quite readily after the addition of a few crystals of sodium chloride. Collect the precipitate on a hardened filter paper, wash with acetone, and dry over sulfuric acid.

Group-specific substances are now available commercially from several sources.

PROFESSIONAL DONORS

Professional donors should carry a book in which are inscribed the name, residence, age, result of serologic tests, and the blood group. Each withdrawal of blood should be entered in this book, together with the date.

The question of just when blood can safely be taken from the same person the second time has been much discussed. The rate of hemoglobin regeneration in blood donors has been studied. Apparently donations of 500 ml. blood at intervals of 4-5 wk. are not harmful to male donors, but in female donors anemia often develops under similar conditions. Blood should not be taken more frequently than once every 8 wk. (N.I.H. requirements). Hemoglobin regeneration is slower in female donors than in male donors. The average drop in blood hemoglobin after the removal of 555 ml. blood is 2.3 gm., and the average time required to replace this amount is 49.6 days. With a smaller donation and a smaller drop in the blood hemoglobin, the recovery period is shorter. Subsequent donations may be given with safety as soon as the blood hemoglobin has returned to its original level. A minimum interval of 8 wk. should be allowed between blood donations, provided that at that time the hemoglobin of the donor has returned to normal. Administration of 1 gm. iron and ammonium citrates per day increases the daily hemoglobin regeneration by 49% and shortens the recovery period from 49.6 days to 35.2 days during the first period of its administration. It has progressively less effect after subsequent blood donations.

DISEASES THAT MAY BE TRANSMITTED BY TRANSFUSION

Syphilis: The donor must be free from acute or chronic disease; more especially must it be proved as accurately as possible that he does not have syphilis. A physical examination should be made by a physician, and serologic tests including preferably the VDRL and a standard complement fixation test such as the Kolmer must be made. It must be remembered that although a negative serologic reaction for syphilis is not complete proof that the donor is free of the disease, a positive reaction is sufficient reason to reject a candidate for donor.

There are many cases on record in which syphilis has been communicated to the recipient because of the use of a syphilitic donor's blood. In the case cited by Wildegans,[273] the patient showed widespread syphilitic skin eruption 10 wk. after the transfusion. The case of Moritsch and Wittman[274] details transmission of syphilis from a donor who had a negative complement fixation reaction but who later was proved to have had a primary syphilitic lesion at the time of the transfusion, which further points up the importance of the physical examination.

The syphilitic infection is transmitted directly into the blood stream without development of any primary manifestation. As a rule, the incubation period is 4-10 wk. and is usually followed by a vigorous and typical roseola (syphilis d'emblée).

Professional and voluntary blood donors, who may be exposed to infection with syphilis during intervals between transfusions, should have serologic tests made for syphilis prior to every blood transfusion regardless of the frequency of the donations and relation to the recipient. As pointed out by Rein et al.,[275] blood donors' assertions of noninfection cannot be depended upon and should not be taken seriously.

With the advent of blood banks, the problem of transmission of syphilis through blood transfusions became somewhat simplified because (1) ample time is available for carrying out tests for syphilis prior to using the donor blood and (2) blood stored at refrigerator temperature for 72 hr. is possibly incapable of transmitting syphilis even though obtained from a donor whose freshly drawn blood is infectious.

Turner and Diseker[276] conducted experiments on the duration of infectivity of *Treponema pallidum* in citrated blood stored under conditions obtaining in blood banks. They added *T. pallidum* to citrated blood from both human beings and rabbits and determined the infectivity of the mixture for rabbits after various periods of refrigeration. Blood from syphilitic rabbits, known to be in an infectious stage, was likewise tested. Blood from human beings with early syphilis was not tested because even under the most favorable conditions rabbits are infected with difficulty with such material. The strain of *T. pallidum* used in the experiments was well adapted to the rabbit. The experiments showed that under the conditions obtaining in blood banks, syphilitic treponemas probably undergo progressive deterioration during the storage period. Even when large numbers of virulent treponemas were added to citrated whole blood, the mixtures were not infectious for normal rabbits after storage for 3 or more days. After storage for 1 and 2 days, although viable organisms were still present, they were evidently considerably reduced in numbers or in virulence since the incubation period of the lesions resulting from the inoculation of the same amounts of the stored mixture was significantly prolonged, and in some instances the inoculated animals escaped infection. Turner and Diseker concluded that the infectivity of syphilis treponemas in citrated whole blood rarely persisted for longer than 3 days at refrigerator temperature.

Bloch[277] obtained infection with material stored for 72 hr. but did not demonstrate survival for 96 hr. He concluded that there is definite risk of transmitting syphilis when using blood from patients with early syphilis if the blood has been preserved for 72 hr. or less in a blood bank.

Although the risk of transmitting syphilis through the use of stored syphilitic blood diminishes rapidly after 96 hr., it is still unwise to use blood from persons with known early syphilis.

As Gradwohl[278] stated, "these experimental observations confirm the statements made above and should cause one to pause in listening to speculative platitudes from those uninformed in experimental data that syphilitic blood when stored is safe and can be given with impunity. The axiom that no syphilitic blood should ever be given in transfusion, whether fresh or stored, still obtains."*

Viral hepatitis: Hepatitis, apparently of viral origin, may be transmitted by blood transfusion. The incidence of supposedly healthy but nevertheless infectious donors is approximately 0.2-0.5%. The incubation period varies from 2 wk. to 6 mo. Cases with short incubation periods are thought to be due to virus A, infectious hepatitis, whereas those with incubation periods of 6 wk. or longer are thought to be due to virus B, serum hepatitis. In practice it is difficult to classify individual cases. The incidence of hepatitis is reduced by careful history and by exclusion from future blood donations of all donors whose blood might have induced hepatitis. It is difficult to distinguish between hepatitis due to the blood or blood fraction previously administered, natural infection, and use of inadequately sterilized syringes and needles. The necessity for careful sterilization of syringes and needles or any skin or mucous membrane-penetrating device should be constantly emphasized to reduce the incidence of hepatitis. Use of expendable apparatus and needles will obviate this.

On the subject of hepatitis carriers, Unger[279] suggested that donors be asked the following questions, and that if the answer to any of them is "yes," the donor should be rejected: (1) Have you ever been jaundiced? (2) Have you ever had a prolonged, febrile, undiagnosed illness? (3) Have you recently received an injection of whole blood or blood products? Unger also stated that since **blood contaminated by the hepatitis virus cannot be detected,** the blood bank at New York University Medical Center places the following notice on each bottle of blood issued: "Despite careful selection of donors, this blood may contain the virus of homologous serum hepatitis."

Malaria: Refer to p. 1585.

Other diseases: Diseases such as rubella and brucellosis may rarely be transmitted by blood transfusion.

UNTOWARD REACTIONS FOLLOWING BLOOD TRANSFUSIONS[280, 281]

Up to the time of the appearance of the publications of Landsteiner[282, 283] there had been repeated grave manifestations of agglutination and hemolysis from the mixture of incompatible bloods. His work showing the blood groups of human beings laid the foundation upon which has been built the relatively safe and secure methods of blood transfusion as practiced today. Reactions sometimes occur because of errors in blood grouping and the consequent transfusion of blood of an incompatible group. Wiener et al.[284] discussed their experience with reactions in more than 3000 transfusions, dividing transfusion reactions into 2 main groups: (1) those due to some specific incompatibility between the blood of the patient and that of the donor and (2) those due to nonspecific agents, the latter constituting the vast majority.

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*From Gradwohl, R. B. H.: Clinical laboratory methods and diagnosis, ed. 3, St. Louis, 1943, The C. V. Mosby Co.

Attention has been called to the danger of using group O blood indiscriminately as a universal donor. Levine and Mabee[285] pointed out that in group O donors with high isoagglutinin titers the dilution of donor's serum in the patient's circulation might not be sufficient to prevent hemolytic reaction; in fact, cases have been reported of hemolytic reactions following use of such dangerous universal donors. It is reasonable to believe that such instances of reactions are certainly high when universal donors are used routinely.

Transfusions of blood containing the Rh agglutinogen to patients lacking the agglutinogen, with subsequent anti-Rh agglutinin development, is definitely a factor in many blood transfusion reactions. This has been fully explained on pp. 1507ff.

All transfusion reactions should be investigated to determine avoidable causes, and this is particularly true when the patient may require additional transfusions.

Hemolytic reactions

Hemolysis in vivo follows when incompatible blood antigens and antibodies are present in the circulation. There is a relative decrease in red cells and release of free hemoglobin, which colors the plasma or serum and may appear in the urine. Free hemoglobin is converted to bilirubin, as shown by increased serum bilirubin levels, and jaundice may develop. Kidney disease with lower nephron nephrosis is a possible complication and is characterized by oliguria, hematuria, anuria, and an increased blood urea. In extreme instances death follows.

Hemolytic reactions may occur after administration of a small amount of blood, but they occur usually after introduction of 50 ml. or more of blood. The patients complain of tingling sensation or a sense of great discomfort and anxiety, fullness in the head, precordial oppression or a constricting feeling in the chest and difficulty in getting their breath, and severe pain in the back of the neck, chest, and especially in the lumbar region. Signs of collapse follow, with rapid feeble pulse, cold clammy skin, marked flushing of the face, dyspnea, cyanosis, drop in blood pressure, dilatation of the pupils, and nausea and vomiting.

In less severe instances there may be very little complaint on the part of the patient. When a hemolytic reaction is suspected, the red count, test for free hemoglobin in the blood, serum bilirubin, and urine volume and free hemoglobin tests prove or disprove the suspicion. Rise in bilirubin values and a positive direct Coombs (antiglobulin) test during the first 24 hr. after transfusion are important in diagnosis.

The cause of a hemolytic reaction should be ascertained when possible. The pretransfusion blood specimens of the donors and recipient and fresh specimens from the donors and recipient should be retested for blood group and Rh-Hr types and antibodies and for antibodies of other systems when other tests prove negative. Cross-matching tests should also be repeated, including antiglobulin tests.

One possible cause of a hemolytic transfusion reaction may be an error in identification, which could occur at the time of withdrawal of the blood specimen. Such errors are avoided if the test tubes are carefully marked with full identification with a clearly inscribed and firmly attached label. Identification of such specimens by casual wax pencil marking on glass should be prohibited. Blood banks and laboratories must insist upon properly identified material. To avoid this error, careful identification should be made of the blood in pilot tubes, and such tubes should be firmly attached to containers to receive the blood before the phlebotomy takes place. It is also imperative that blood be administered only after careful inspection of the statement of compatibility to be sure that the blood is given to the correct patient. Awareness of possibility of error of identification lessens the incidence of such reactions.

Nonspecific reactions

Allergic reactions: Allergic reactions produce hives, edema (angioneurotic edema), or a tendency to asthma. Donors with active extreme allergic manifestations should be avoided. The cause of most allergic reactions is not well understood. They may be associated with fever and even with chills, but more commonly there is little increase in temperature. Adrenalin or antihistaminics are often used in treatment. If a recipient has had an allergic reaction from a blood transfusion, use of antihistaminics administered orally or by injection beforehand may possibly be helpful in preventing a subsequent similar reaction.

Pyrogenic reactions: Pyrogenic reactions are associated with a sharp temperature rise and often with chills and are usually transient. They were much more common in the past. The use of expendable equipment has greatly reduced the incidence of these reactions. Careful cleansing of all equipment with which blood comes in contact and prompt sterilization of such equipment after cleansing have also greatly reduced pyrogenic reactions due to contaminating chemicals or bacterial toxins. An occasional reaction may be due to the use of intravenous administration needles that have received only casual cleansing. Recent evidence points to the presence of leukoagglutinins in recipients as the cause of some pyrogenic reactions. Such reactions follow multiple transfusions. In such cases administer packed red cells prepared free of white cells.

Cardiopulmonary reactions (volume, circulatory overload): Occasional patients, particularly those with cardiac or pulmonary disease, display intolerance to rapid infusion of such materials as blood which persist in the patient's circulation. The symptoms commonly seen are those of pulmonary edema together with a rapid feeble pulse and a shocklike state. Blood transfusion should be given slowly or in lesser volume per infusion, or packed red blood cells should be used when cardiac or pulmonary disease is present.

Bacterial contamination: With the use of sterile equipment, careful disinfection of the skin of the donor and patient, and storage in a refrigerator at 2°-10° C., preferably 4°-6°, the chances that multiplication of any bacteria present will take place is minimized. Entry into the bottle during storage is strictly prohibited. Nevertheless, at times bacteria gain access to the blood and may multiply. The most dangerous are gram-negative bacteria which can grow at low temperatures and produce highly toxic endotoxins. Contamination of the blood is suspected if it changes color (bluish black), if a zone of hemolysis is observed in the lowermost portion of supernatant plasma, or if the plasma seems to have unusually large quantities of free hemoglobin when the blood is shaken and the cells are allowed to resettle. Some bacteria metabolize citrate, and clots will develop in the blood. The appearance of clots in blood previously thought satisfactory should arouse suspicion.

In some instances administration of contaminated blood is followed by a persistent shocklike state, with moderate fever and occasionally death. Evidence of hemolysis may be seen. If the remainder of the blood can be saved, with precautions taken to prevent contamination from handling, cultures and smears of the remaining blood may clarify the problem. Strict aseptic technic is important.

Coagulation defects: Hemorrhagic disorders frequently follow use of massive transfusions of **over-age** blood, for example, administration of 10-15 or more units in a 24 hr. period. This has been named "oozing disease" because blood oozes from all cut surfaces. Bleeding may also occur from mucous membranes, sites of needle punctures, and sites of injury; petechiae are sometimes noted.

Thrombocytopenia is a common finding in such patients and is probably due to a loss of platelets at initial bleeding sites, failure of stored blood to replace the lost platelets, and perhaps destruction or removal of platelets by administered blood. The platelet deficiency, however, does not always seem severe enough to cause the bleeding. Deficiencies of other clotting factors and the appearance of a fibrinolysin have been observed in some patients.

The complication is most likely to occur in patients who already have a hemorrhagic diathesis or in those who have liver disease. Treatment is difficult and should include vigorous effort to stop bleeding at local sites together with transfusion of fresh blood that comes in contact only with nonwettable surfaces.

Since transfusion of incompatible blood may also lead to coagulation defects, this cause is often suspected. Keeping this in mind should encourage those responsible to take care that blood used in massive transfusions is, as always, carefully cross matched.

Cardiac arrest: Massive transfusions are sometimes followed by hypotension and cardiac arrest. It is generally believed that this is due to free potassium in stored blood or to depression of **ionized** calcium in the recipient which is bound in nonionized form by excess citrate in transfused blood. The heart's normal pumping function depends on a minimal level of circulating ionized calcium. The disorder is often called "citrate intoxication." The liver ordinarily rapidly detoxifies citrate as it enters the circulation; therefore, the disorder is most likely to occur in patients with poorly functioning livers or in circumstances when transfused blood is unable to reach the liver in adequate amounts.

Treatment is somewhat empirical and depends on the intravenous administration of ionized calcium, e.g., $CaCl_2$, in periodic injections. The disorder may be avoided by use of uncitrated blood, e.g., blood collected in heparin or over a cation-exchange resin. The use of uncitrated blood, however, introduces other complications so that citrate remains the anticoagulant of choice for the vast majority of transfusions.

Overheating of blood: Blood should not be warmed to 37° C. before administration, because if the temperature of even a small portion of the blood is raised above 37°, toxic changes ensue. Overheated blood induces pyrogenic and even hemolytic reactions.

Outdated blood: Administration in a short time of large quantities of blood approaching expiration date may introduce into the recipient appreciable quantities of at least partially degenerated red cells that are rapidly hemolyzed. This may result in release of free hemoglobin in quantities in excess of the bilirubin-clearing mechanisms of the recipient, and a temporary, usually mild degree of jaundice may be observed.

Pharmaceutical incompatibilty: No drugs or solutions should be mixed into blood or injected through the walls of intravenous administration sets while blood is being infused unless it is certain that no pharmaceutical incompatibility exists; e.g., calcium-containing preparations induce clotting. Low pH vitamin preparations may cause denaturation. Tincture of iodine used wastefully in preparing the donor's skin or sterilizing a stopper may be introduced into the blood and denature it.

Other reactions: Bank blood, particularly with ACD solution as anticoagulant, has a tendency to form minute masses of fibrin on standing. Such blood is perfectly safe if administered through a filter to avoid entrance of such particles into the recipient. The finer mesh filters, 75-200 holes/sq.in. are preferable. On occasion, bloods of different groups may be administered to a single recipient, e g., A and B bloods to an AB recipient. In these instances such bloods may react with each other within the intravenous administration set. The problem is avoided by changing the intravenous administration equipment when the donor blood group is changed.

TECHNIC OF WHOLE BLOOD TRANSFUSION

Direct method of blood transfusion

There are various methods of direct blood transfusions dependent upon the type of apparatus used. The first modern method for blood transfusion using an apparatus was that described by Unger.[258-288] This apparatus permitted withdrawal of blood from the donor and simultaneous injection of saline into the patient. A stopcock was then turned and the reverse situation resulted; blood could be injected into the patient and saline simultaneously injected into the donor. It was this immediate flushing with saline of that part of the instrument not used for the aspiration or injection of blood that ensured freedom from clotting.

Another method is that using multiple glass syringes. This method employs a battery of about twenty 20 ml. glass syringes, sterilized and placed on a sterile table close to the donor. An assistant inserts a needle into the donor's vein, and the operator inserts a needle into the recipient's vein. The first 20 ml. syringe is half filled with warm saline, i.e., about 10 ml., and 10 ml. blood are withdrawn from the donor. The syringe is quickly disconnected and connected to the needle in the recipient's vein, and the first syringe material is injected. While this is being injected, the assistant takes the next 20 ml. syringe, fills it with blood, passes it to the operator, who in turn injects it. In this way the operator, by counting the number of syringes alongside his table, sees at all times just how many ml. blood he has injected.

Indirect method of blood transfusion

The best method of indirect blood transfusion is one that makes use of vacuum bottles, a closed circuit to prevent bacterial contamination, and expendable tubing, needles, and other equipment.*

The use of rubber tubing in blood transfusion is now obsolete. We have therefore eliminated all reference to directions for cleaning and sterilizing such tubing.

The blood equipment consists of Transfuso Vac units, Plasma Vac units, Plastic Cube-Pac units, and plasma separation units, in addition to Plexi-

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*Such equipment is manufactured by Baxter Laboratories, Inc., Morton Grove, Ill.; by Abbott Laboratories, Chicago, Ill., and by Cutter Laboratories, San Francisco, Calif. The Baxter equipment is described here. See Figs. 497-505.

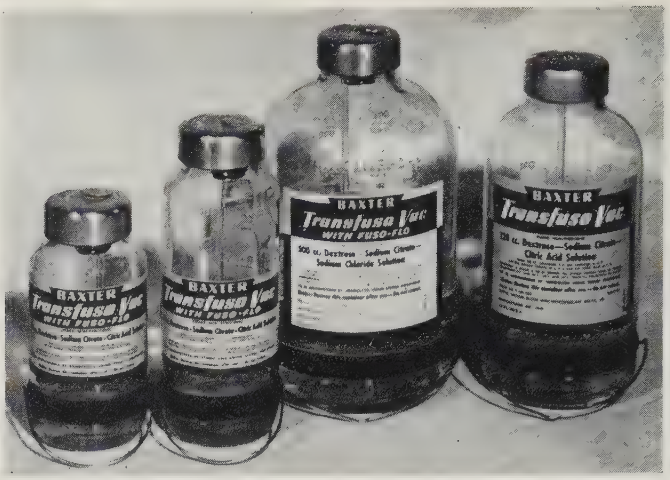

Fig. 497. Transfuso Vac containers. Blood collected in these containers may be stored up to 21 days and administered as whole blood, or plasma may be prepared from the stored blood. The remaining cells may be used for red cell infusion. Capacities of containers are, from left to right, 150, 250, 1000, and 500 ml. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

tron blood collection sets, aspirating sets, blood and plasma administration sets, and accessories designed for blood banking procedures.

The Transfuso Vac container (Fig. 497) is a vacuum unit for collecting, storing, and administering blood and for collecting blood for eventual preparation of plasma. Blood can be administered from the same unit into which it was originally collected by plugging a Plexitron blood administration set through the closed hole outlet of the stopper. If collected blood is not used for whole blood transfusion, the supernatant plasma can be recovered in a Plasma Vac unit. Transfuso Vac units are available with anticoagulants in varying amounts for the collection of 150, 250, 480, and 500 ml. blood, with or without the Fuso-Flo stoppers for preliminary straining and with or without internal silicone coating to provide a nonwettable surface.

The Fuso-Flo stopper provides preliminary straining of blood or plasma prior to filtration in the administration set and helps to promote smooth, uninterrupted flow and full utilization of blood. See Fig. 504.

Collection of blood, using expendable units

Preparation of donor's arm: The operator must wash his hands thoroughly before venesection.

1. Place a blood pressure cuff on the donor's arm, setting the pressure at slightly below diastolic level; then prepare the donor's arm.
2. Select the vein most suitable for puncture and prepare the site as follows:
   - (a) Use an acceptable soap or detergent to cleanse the skin, rubbing the skin vigorously in a circular fashion. Allow 1 min. for this step.
   - (b) Remove the dirt and fat with a solution of 10% acetone in 70% alcohol.
   - (c) Treat the skin with 3.5% alcoholic solution of iodine. Prepare the puncture site from the center outward so as to avoid contamination. As soon as the iodine is dry, remove it with a solution of 10% acetone in 70% alcohol or a saturated solution of sodium thiosulfate in 70% alcohol.
   - (d) Anesthetize the site for insertion by injecting 1 or 2% procaine intradermally. Take care that donors are checked for possible sensitivity to the drug before administering it.
   - (e) Leave a sterile alcohol or dry sponge over the proposed puncture site and do not remove until the needle is ready to be inserted into the vein.
   - (f) Do not touch the skin again once it has been prepared. Additional exploration of the area may be made after the needle has been inserted beneath the skin.

Collection of blood: The needles must be either heat-sterilized or, preferably, should be expendable, and no needle must be used on more than one donor. These precautions are necessary to prevent possible transmission of homologous serum hepatitis.

1. Tear the metal seal from the Transfuso Vac, lift off the metal disk, and remove the top plastic diaphragm aseptically (Fig. 498, *A*). Clamp the Plexitron tubing of the donor set.
2. Insert the needle of the donor set perpendicularly through the sealing diaphragm and stopper at the point marked "X" on the top of the Transfuso Vac. Immediately after insertion, recheck the clamp on the tubing to make certain it is still closed.
3. Insert the needle in the donor's vein through the anesthetized skin with the bevel of the needle up.
4. Loosen the clamp. If the blood does not flow immediately, close the clamp and improve the position of the needle. Improper placement of the needle may cause collapse of the tubing. It will be expanded again by flowing blood when the needle is properly placed in the vein. This is a good check on the needle position. When the blood is flowing freely, slowly open the clamp further. The flow of

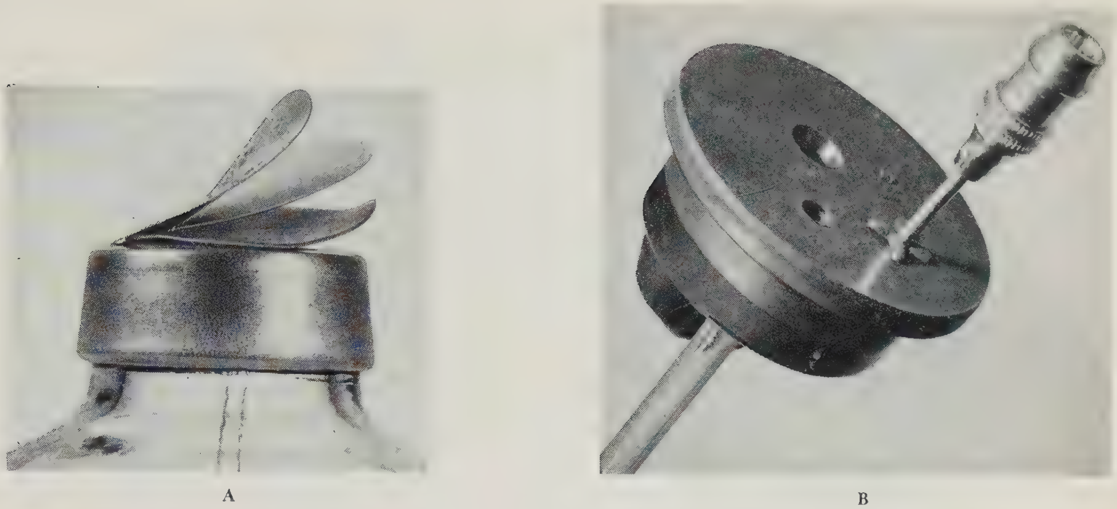
A B

Fig. 498. A, Closure of Transfuso Vac and Plasma Vac containers. Note the metal identification disk under which is a plastic disk which protects sterility and a thin rubber disk to protect the surface of the stopper. **B,** Baxter blood and plasma container stopper. The puncture position is marked with an "X." This thicker portion of the stopper will reseal after use so that the vacuum is retained after the blood unit is filled. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

blood gradually increases until the desired rate is established. The average rate is between 75 and 125 ml./min. The usual causes of insufficient flow of blood are (a) the needle is not in a good position in the vein and (b) the pressure in the blood pressure cuff is too high or too low. Occasionally, a better flow can be maintained if the donor slowly opens and clenches the hand.

Note: If it becomes necessary to remove the needle from the vein, always close the clamp before removing the needle. After the needle has been removed from the vein, hold it as high as possible above the clamp and very slowly open the clamp, allowing the blood remaining in the tubing to be drawn into the bottle. Instantly close the clamp when the blood is entirely out of the tubing. Reinsert the donor needle and slowly reopen the clamp so as not to collapse the vein.

5. When the desired amount of blood has been drawn, clamp the tubing. Remove the blood pressure cuff and withdraw the needle from the vein. Remove the needle from the Transfuso Vac and salvage the blood from the tubing for serologic study by opening the clamp and allowing the blood to run into the sterile pilot tubes, one with and one without anticoagulant. Shake the tube containing the anticoagulant. **Note:** Some blood banks do not use the pilot tubes with anticoagulant.
6. Agitate the Transfuso Vac a minimum of 3 min., assuring adequate mixing of the anticoagulant and blood, thus safeguarding against clotting.

To administer whole blood

1. Invert the Transfuso Vac several times to resuspend the blood cells.
2. With a pair of sharp scissors cut the rubber diaphragm at the extreme edge, farthest from the air tube.

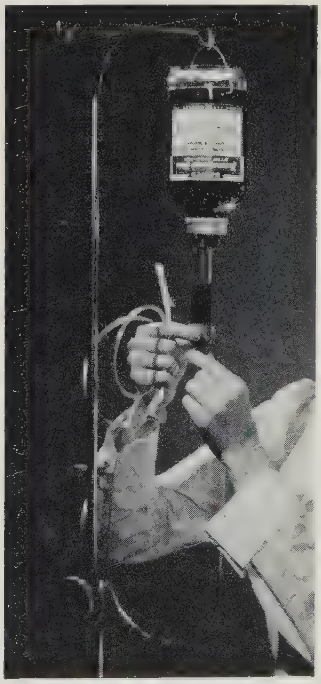

Fig. 499. Drip device of Plexitron administration set is filled with enough blood to cover the filter. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

3. Tear the diaphragm straight across, exposing both holes in the rubber stopper.
4. Close the clamp on the Plexitron tubing.
5. Insert the drip device of Plexitron administration set into the bottle of blood, suspend the bottle by its bail, and fill the drip device with blood as shown in Figs. 499 and 500 *A.*
6. Establish the blood level in the drip device;

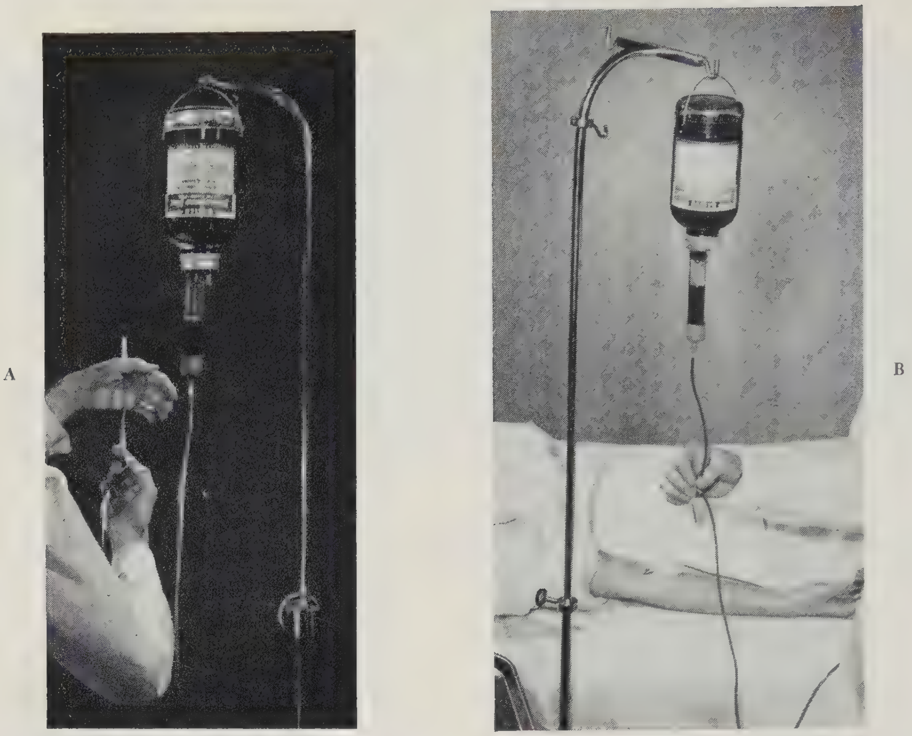

Fig. 500. A, Opening clamp to fill remainder of set before attaching vein needle to nylon needle adapter. This is done after the blood level in the drip device of Plexitron administration set has been established. **B,** Adjusting clamp to control rate of blood administration. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

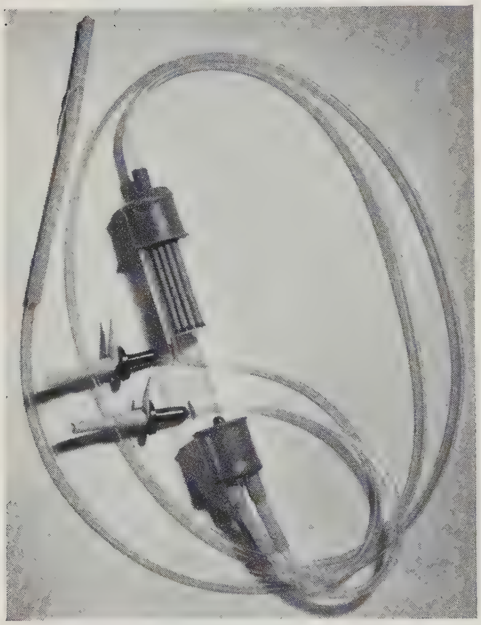

Fig. 501. Sterile expendable Plexitron Y-type set for administering blood, plasma, or serum with solutions, alternately or simultaneously. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

then open the clamp to fill the remainder of the set and to remove all air from the tubing.

7. Attach the vein needle to the nylon needle adapter and again be sure that there is no air in the needle or the tubing.
8. Make puncture of the patient's vein in the usual manner, after disinfecting the skin, and proceed with the transfusion.
9. Adjust the clamp to control the rate of blood administration (Fig. 500, *B*).

If one desires to start the transfusion with saline or to mix saline with the blood or to follow the transfusion with an injection of saline, connect the Transfuso Vac to the bottle of sterile saline, connecting them with a Y outfit leading into a filter drip. This Y outfit is also expendable. See Figs. 501-503.

Plastic bags for transfusions

A method of collecting blood for whole blood, plasma, platelet, and other blood constituents transfusions, by the use of plastic bags, has been devised. The Fenwal system* consists of standard Blood-Pak units and recipient sets. It provides for collection, storage, and administration of whole blood taken in ACD solution, as well as blood components. The units are available for collection of 500,

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*Fenwal Laboratories, Morton Grove, Ill.

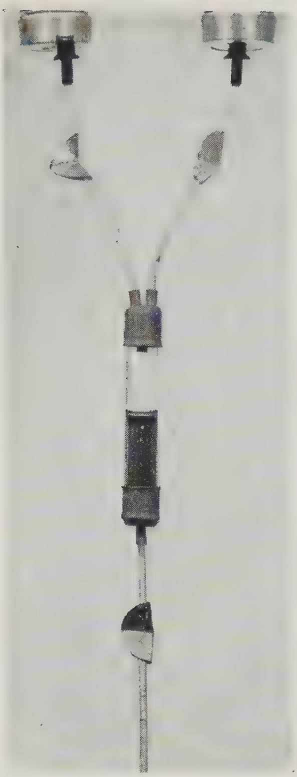

Fig. 502

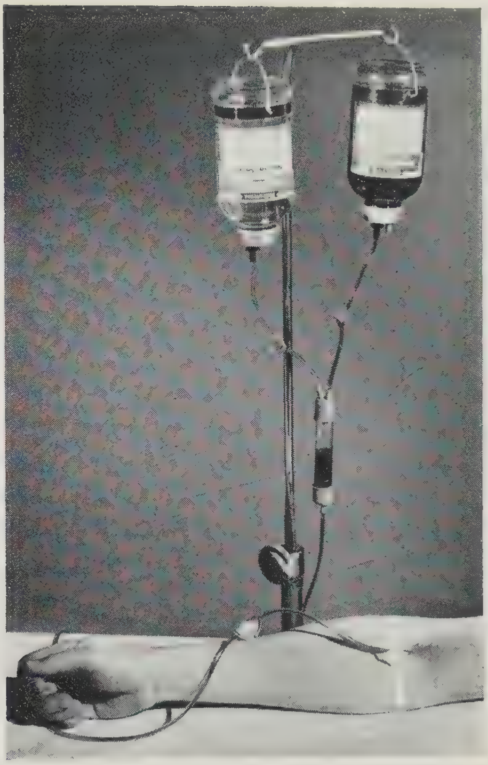

Fig. 503

Fig. 502. Expendable Y-type administration set, with clamps in position. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

Fig. 503. Plexitron expendable Y-type set for administration of blood, plasma, or serum with solutions, alternately or simultaneously. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

450, or 250 ml. blood. One advantage in using plastic bags is that they preserve the blood platelets, which are quickly disintegrated in glass containers.

Collection of blood:

1. Place donor in the position preferred and suspend the unit on a spring donor scale or stand. If the spring donor scale is used, adjust it for tare weight of the unit. Place a loose overhand knot in the donor tube approximately 4 in. from the needle (Fig. 506, *A*).
2. If the automatic donor scale is used, preposition the weight for collection of the desired amount of blood, suspend the unit, and thread the donor tube through notches in the studs in the lever arm and support block (Fig. 506, *B*).
3. Place a blood pressure cuff with tubes pointed toward the shoulder, with the lower edge of the cuff well above the intended site for venipuncture; then inflate the cuff to 60 mm. Hg and locate the vein.
4. Prepare the skin area.
5. Grasp the needle hub firmly and pull off the Interlock cover.
6. Insert the needle with the bevel side up well into the vein. If desired, the needle may be secured in position with adhesive tape.
7. Release the bead in the juncture of the donor tube and the bag to permit the blood to flow. Squeeze the tubing above the bead between the thumb and forefinger. This moves the bead completely into the unit (Fig. 506, *C*).
8. Mix the blood and anticoagulant, beginning as soon as blood flow has been established, elevating the bottom of the unit gently several times during collection. Do not put any tension on the tubing which might disturb the needle.
9. If the spring donor scale is used, collect the blood to the desired volume. If the automatic donor scale is used, the weight of the unit depresses a lever arm which closes the donor tube and stops the flow of blood.
10. Close the donor tube near its attachment to the unit, using a clamp (Fig. 506, *D*), release the pressure in the cuff, and remove the cuff. Apply a sterile dressing and withdraw the needle from the vein. It is customary to have the donor elevate the arm without flexing the elbow and continue digital pressure to prevent oozing.
11. Insert the needle through the stopper of the evacuated pilot tube to obtain blood without

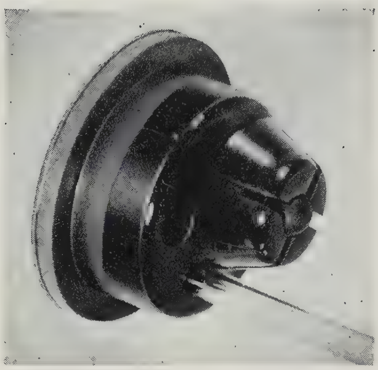

Fig. 504

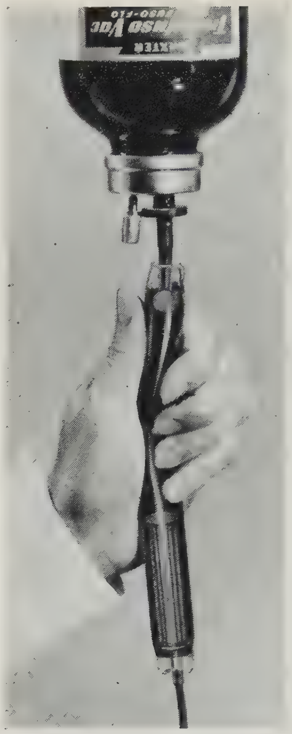

Fig. 505

Fig. 504. Fuso-Flo stopper available for Transfuso Vac containers. Although it does not replace the blood filter, it acts as a preliminary strainer for blood or plasma during administration and prevents the fibrin of stored blood from retarding the flow. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

Fig. 505. Baxter Plexitron combination administration set and pressure pump, which enables administration of blood at the normal rate or rapidly under pressure. The rate of administration is determined by pumping action. This pump **cannot pump air,** since it operates with fluids. It contains a filter that permits rapid administration through mesh fine enough to remove particulate matter. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

anticoagulant for serologic testing and blood grouping (Fig. 506, *E*). All the blood in the donor tube must be evacuated. Pull the knot until it is "white tight"; then cut the donor tube between the knot and the needle, withdraw the needle from the evacuated pilot tube, and discard the cut portion and the needle. A seal may be applied, according to the manufacturer's direction. Seal at mark X nearest the needle and sever in the mid-portion of the seal. Crimp the tubing with mechanical pressure and sever on the needle side of the clip.

12. Mix the blood thoroughly, lay the bag flat, and release the clamp. Permit citrated blood to reflux into the donor tube, strip, and refill the donor tube once.
13. Seal off all segments at the X points. Insert through slits on either side of the bag. Always start the seals at the distal end of the tube and work toward the bag. If the seals are started proximal to the bag and then made in the opposite direction, the segments might rupture due to pressure buildup.
14. The unit may be placed in an upright position in a plastic holder, and it must be refrigerated at once.

Other equipment is available, such as Hemolator,* and special anticoagulants are also supplied by the manufacturer.

Transfusion procedure:

1. Remove the recipient set and prepare for assembly.
2. Mix plasma and cells thoroughly by inverting the plastic unit at least 12 times.
3. Close the control clamp tightly on the lower end of the plastic tubing.
4. Grasp the end of the coupler cover, push toward the flange, and then twist to loosen (Fig. 507, *A*). Do not remove.
5. Grasp the tabs closest to the side of the bag and pull them apart. Fold back to expose sterile outlet tube (Fig. 507, *B*).
6. Hold the plastic unit between the thumb and forefinger over the folded tabs, remove the cover from the coupler, insert the coupler into the outlet tube, and twist until it is all the way in (Fig. 507, *C*).
7. Remove the needle adapter cover.
8. Hold the unit in an inverted position, open the control clamp, and squeeze the bag to fill the filter chamber with blood.

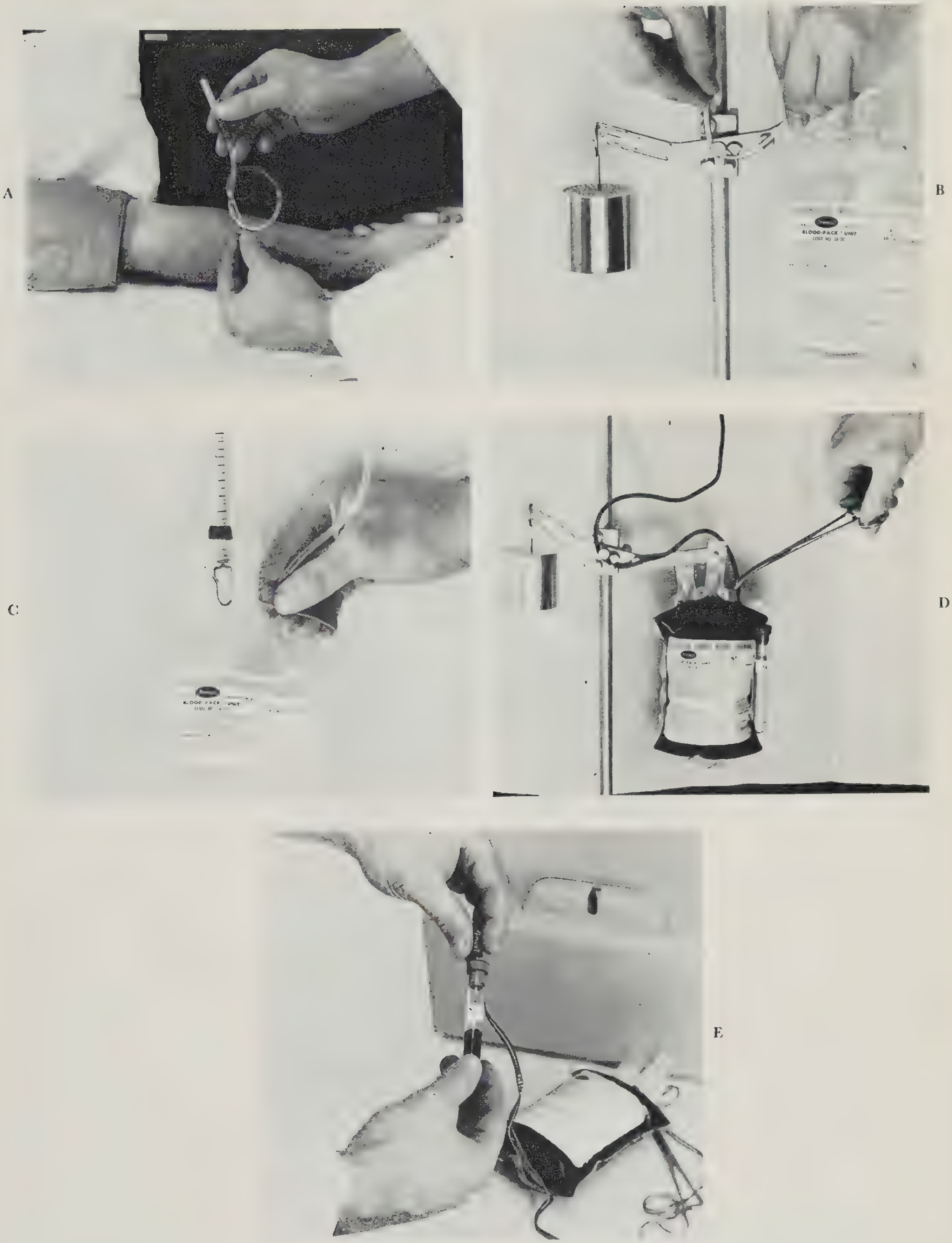

Fig. 506. Collecting blood by use of plastic bags. **A,** Placing loose overhand knot in donor tube. **B,** Pre-positioning weight for desired quantity of blood. **C,** Moving bead into Blood-Pak unit. **D,** Clamping off donor tube. **E,** Collecting clotted blood for serology and blood grouping. (From Technical Manual, Morton Grove, Ill., 1962, Fenwal Laboratories.)

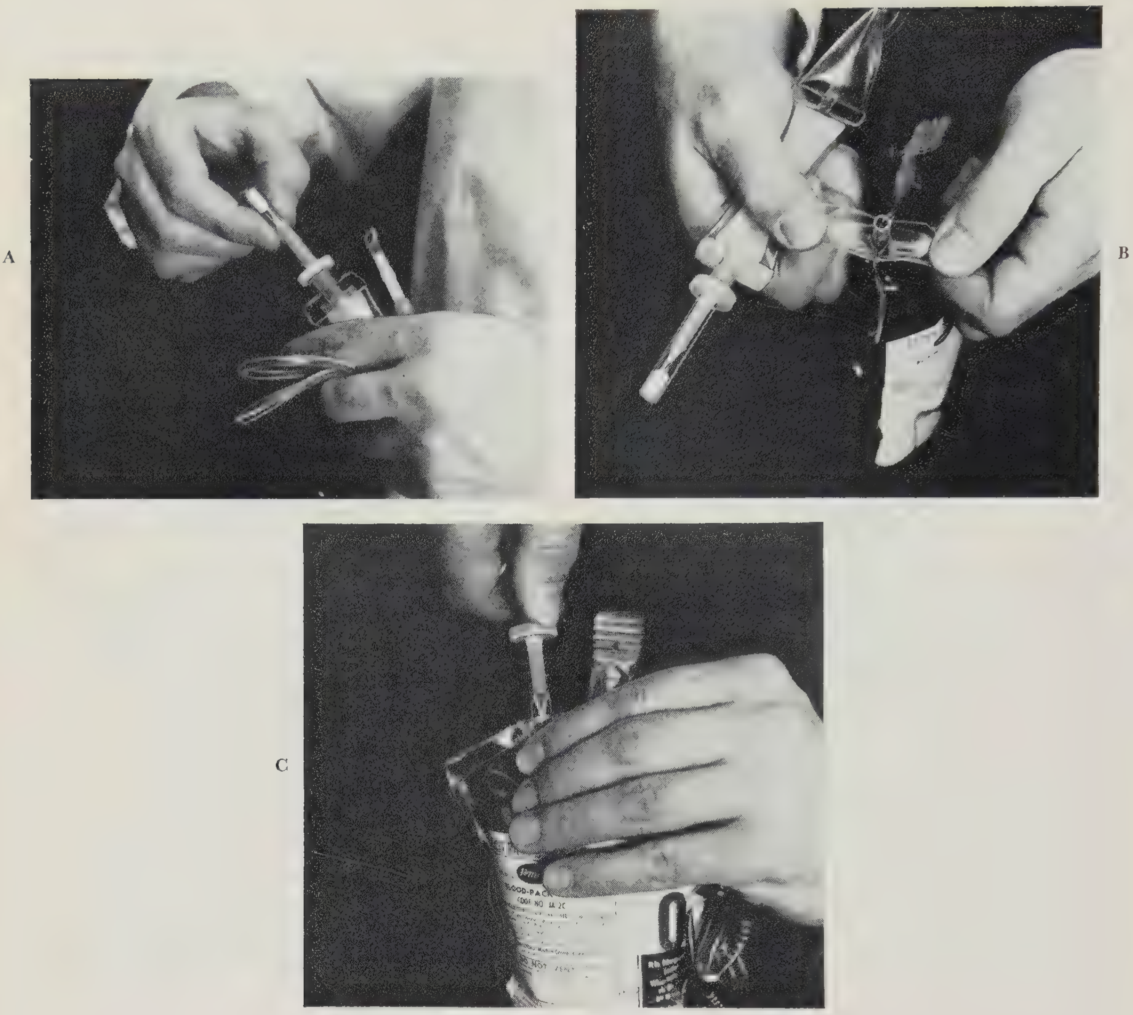

Fig. 507. Transfusion procedure, using blood collected in plastic bags. **A,** Loosening coupler cover. **B,** Exposing sterile outlet tube. **C,** Inserting coupler into outlet tube. (From Technical Manual, Morton Grove, Ill., 1962, Fenwal Laboratories.)

9. Close the control clamp and suspend the unit.
10. Squeeze the drip chamber slightly; then release it. This fills it about ¼ full.
11. Attach the sterile needle. Release the clamp to fill the tubing with blood to the needle point; then close the clamp tightly.
12. Insert the needle into the recipient's vein in the usual fashion after disinfecting the skin, adjust the control clamp for desired flow rate, and continue with the transfusion.

Blood component separation: Standard centrifuge cups for 650 ml. bottles may be used for all Fenwal blood pack units of 300, 500, or 600 ml. A table of r.p.m. and recommended g's is supplied with the units. Full directions for preparing blood platelets, packed red cells, plasma, etc. are included with the apparatus.

PRESERVATION OF WHOLE BLOOD IN THE FROZEN STATE

Whole blood modified with addition of dextrose and lactose, alone or in combination, to obtain a 0.2M concentration of lactose, a 0.7-1.2M concentration of glucose, or an additive molarity of 0.6 with both sugars may be frozen in about 10 sec. or less by immersion in CO_2-ethanol mixtures at –60° to –78° C.[289] It is then thawed equally rapidly at +37° to +40° C., and 95% of the erythrocytes are recovered intact. Without the sugars recovery of human erythrocytes frozen and thawed under similar conditions averages 29.3%. Recovery and posttransfusion survival of erythrocytes depend on the temperature of storage. At –58° C. recovery and survival deteriorate rapidly, at –70° C. recovery rate is fairly well maintained for about 40 days, but the post-transfusion survival is unsatisfactory after 3 weeks of storage, and at −93° C. ± 2° C. satisfactory recovery and survival are maintained for at least 6 mo.

BLOOD FROM CADAVERS FOR TRANSFUSION—HISTORICAL

Considerable interest was evoked by the announcement of Judin, chief surgeon of Sklifasovski Emergency Hospital in Moscow, who within 3 yr. had performed more than 300 blood transfusions, taking the blood from dead individuals, storing it, and then injecting it into living patients. Judin's work has been referred to by Gosset, chief surgeon of Salpetriere Hospital in Paris, in a publication entitled "La transfusion du sang de cadavre a l'homme."

In Moscow, whenever an acute accidental death occurred from suicide, homicide, or in an industrial accidental way, the dead subjects were immediately brought to one hospital where their entire venous blood was removed and stored. The cadaver was strapped on his back on the operating table, covered with sheets and sterile drapings, just as would be done in the case of a major operation on the living. The neck was left exposed and prepared with iodine and alcohol, etc. The jugular vein was exposed and 2 slits made into the vein, one for a cannula to receive blood from the head, the other to receive blood from below. These cannulas were connected with glass sections and rubber tubing, terminating in sterile glass jars stoppered with cotton. No citrate was added. The blood clotted, was placed in a refrigerator, and within 2 hr. the blood was fluid. The clots had autolyzed. The operating table was tipped forward at a sharp angle and blood permitted to flow by gravity into the jars. The filled jars were kept at refrigerator temperature just 1° or 2° above freezing point and warmed in a water bath at 40° C. just before use. Judin kept this blood as long as a month and used it without reaction. Kahn and complement fixation tests were made on the specimens before use.

According to this account of Judin's work, the blood of many cadavers has been tested and found sterile. Of course, the subjects used are persons who have died suddenly, from accidents, suicide, or heart attack. It is claimed that all fresh blood from this type of death is free from bacteria. This raises a question, for in 1904 Gradwohl[290] reported an account of a research upon this very question; namely, does the blood of cadavers meeting death from acute accident or from a sudden illness contain bacteria? This was in reply to a publication of Simmonds,[291] who claimed that in a bacteriologic examination of the cardiac blood of 1200 subjects, he found a streptococcus in 95% of all cases, autopsy being performed from 12-36 hr. after death. Canon[292] later claimed that Simmonds' work was in error in that cardiac blood alone was examined and that if at the same time venous blood had been examined, it would have been found that venous blood was sterile, even with streptococci in the heart blood. Grahwohl's work was undertaken in an attempt to settle this point, and so 50 cases were selected from his service at the Coroner's office, in the main, gunshot wounds of the chest, fractures of the skull, 10 cases of valvular leak, 3 cases of rupture of aortic aneurysm, 6 cases of nephritis, a few cases of purulent peritonitis from abortions, etc. The time elapsing from death to the moment of autopsy performance varied from as little as 2 hr. to as long as 10 hr. In 78% of these cases bacteria of various kinds were found in the cardiac blood; in only 3 cases were bacteria found in blood from the median basilic vein. The bacteria found were streptococci, staphylococci, *Proteus vulgaris, Escherichia coli,* pneumococci, and pyocyaneus. Gradwohl concluded that there is a rapid invasion of the cardiac blood soon after death, from neighboring organs, and that bacteriologic results therefore from cardiac blood in the cadaver must be weighed in the balance before being accepted as causative factors in deaths of such cases.

These findings give us pause in accepting the Russian announcement, which, although emphasizing that the blood from the jugular veins is free from bacteria, still does not take into account that blood drainage in Judin's technic, coming from the region of the heart as well as from other parts, makes it possible to introduce these postmortem invaders into the recipient end of the transfusion. The modern blood bank supplants this Russian procedure.

DIFFERENTIAL AGGLUTINATION[293] —THE FATE OF TRANSFUSED CELLS

The principle of the differential agglutination test is to take a sample of the patient's blood after a transfusion with universal donor blood and test it with serum capable of agglutinating the patient's cells. Since the donor belongs to group O, his cells will not be agglutinated by the specific antiserum. For example, if an individual of group A is given group O blood, a count may be made of those cells not agglutinated by anti-**A** serum after the transfusion. Group O cells are not agglutinable by anti-**A** serum, but the patient's group A cells are, and thus it may be assumed that any cells not agglutinated are the transfused cells. It must be remembered, however, that in normal blood approximately 50,000 cells/cu.mm. are not agglutinable by specific antisera, and this must be considered when the test is performed. The method of tracing the fate of transfused red blood cells was first used by Todd and White[294] in cattle. Ashby[295] used the method for the first time in man.

When the patient receives blood of his own group, the differential agglutination test may be made only provided the M-N types of the donor and recipient differ. The results with anti-**M** serum are more striking than when group O blood is used for patients of groups A, B, and AB, for, if tests are made using anti-**M** serum on blood containing the M agglutinogen, very few cells remain unagglutinated as contrasted with the 50,000/cu.mm. not agglutinable by anti-**A** or anti-**B** sera. For example, if OMN blood is transfused to an ON patient, when tests are made using anti-**M** serum on the patient's blood after the transfusion, only the donor's MN cells are agglutinated; any unagglutinated cells are the patient's. If the donor is ON and the patient is OMN, the unagglutinated cells in tests with anti-**M** serum are the donor's.

Landsteiner et al.,[296] Wiener,[276] and Dekkers[298] established that the blood cells of the donor survive in the patient's circulation for periods up to 3-4 mo. in the usual case where the patient is transfused with fresh compatible blood either from a universal donor or from a donor of the homologous group. Wiener and Schaefer[299] showed that the survival time of erythrocytes in bank blood is roughly in inverse proportion to the period of storage.

If incompatible blood is transfused, it remains in the circulation usually for only a short time,[300, 301] so that even if the transfusion is tolerated, at best it is of only transient benefit because of the rapid destruction of blood cells. However, Jervell[302] found incompatible cells in an infant for more than 6 wk. after a transfusion, and Wiener found group AB cells present in a group O infant 50 days after a transfusion.

Procedure: The method of making the test is to dilute the patient's blood taken before the transfusion with potent specific antiserum, in the usual manner for making a red blood count. Count the number of inagglutinable cells/cu.mm. in the patient's blood.

Repeat this test after the transfusion.

To determine the number of donor cells/cu.mm., subtract the number of unagglutinated cells before the transfusion from the number after the transfusion and record the number/cu.mm. Use formula for figuring red counts on pp. 1103 and 1106.

If the count after the transfusion exceeds the count before the transfusion, it is assumed that the transfused erythrocytes are still present in the patient's blood.

Wiener and Peters[303] applied differential agglutination tests to the study of posttransfusion hemolytic reactions and found that even when patient and donor are of the same blood group, if the bloods are incompatible due to factors other than **A** or **B**, the donor's cells are eliminated rapidly from the patient's circulation, within a period of a few hours or days, rather than in 3 or 4 mo. The diagnosis of hemolytic transfusion reactions using this method rests on the assumption that the donor's blood has been eliminated from the patient's circulation.

PREVENTION OF HEMOLYTIC REACTIONS

No matter how carefully one groups bloods and performs all the tests for compatibility, certain reactions at times seem bound to occur. This is possibly due to the fact that there are as yet certain facts about the problem of intragroup reactions that have not been solved. Accordingly, Wiener et al.[304] suggested the use of a biologic test to supplement those in vitro tests commonly used. The use of a biologic test was suggested first by Oehlecker,[305] who advised the injection of 10 ml. blood, waiting 10 min., and then proceeding with the remainder of the blood if no reaction occurs. This method was not too successful because the reaction is often delayed beyond the 10 min. period of Oehlecker. Wiener[304] therefore recommended the following procedure.

Biologic test

Transfuse 50 ml. of donor's citrated blood. After 2 hr. have elapsed, if no reaction has occurred administer the balance of 100 ml. blood. If a reaction occurs, withdraw 10 ml. blood from the patient and treat it in the same manner as the pretransfusion citrated sample used for the cross-matching test. Centrifuge the citrated blood samples and compare the color of the plasma in the 2 tubes. The syringe and needles **must be dry.**

If there is no change in the appearance of the patient's plasma, proceed with the transfusion of larger amounts of donor's blood. If the supernatant plasma of the posttransfusion specimen is distinctly redder than that of the pretransfusion sample, give no more of this donor's blood.

After 100 ml. of donor's blood have been injected, it is possible to demonstrate the survival of the donor's cells by the method of differential agglutination (p. 1579) when the biologic test is negative, in patients having a red cell count of 2½ million cells/c.mm. or less.

In positive reactions, 2 hr. after the test injection of 50 ml. blood, patient may have a severe chill and rise in temperature, but clinical symptoms may be mild or absent. Place more reliance on the appearance of the patient's plasma which will show a distinct rise in icteric index as compared with the pretransfusion sample.

Chapter 80

Stored blood

ADDINE G. ERSKINE

BLOOD BANKS

The so-called "blood bank"[306-308] was first established in Russia, but the first in America was at the Cook County Hospital, Chicago, Ill. It was eminently successful and has been adopted throughout the world. It is essentially a method of keeping on hand quantities of blood so that the hospital is always prepared to give blood transfusions. Like all banks, it is operated on the principle that withdrawal of blood means substitution of other blood, so that the supply on hand is fairly constant. A blood bank is a useful adjunct for an institution, provided it can afford the equipment and personnel.

Unless a blood bank is properly equipped and under control of responsible personnel and is continuously checked, it is obviously a dangerous addition to any hospital program.

Blood is obtained from relatives and friends of patients who are transfused. There must be direct contact with the donors. Donors should be between the ages of 21 and 59 yr. and in good health; they should be free from syphilis as proved by serology and physical examination; and there should be no history of recent illness, mild or severe; chronic alcoholics should be rejected. Donors who meet these requirements must certify in writing that they release the hospital from all liability, and they must give permission to use their blood as the hospital authorities may elect. The rules governing a donor clinic are as follows:

1. Those in charge must be well trained and well supervised.
2. Simple, uniform technic must be used.
3. Unusual care must be taken in technic to preserve the product.
4. Discomfort of the donor must be reduced to a minimum.
5. Adequate room must be provided and unnecessary delay avoided.

The practice is to draw blood from all donors without preliminary typing or testing. This blood is deposited in favor of the patient for whom it was given. That means that each transfused patient has an account and he may receive blood until his account has been exhausted. If any patient has more than is needed, this may be transferred to the account of some patient who lacked donors. Withdrawing blood from all donors who volunteer prevents the necessity of taking large amounts from any one person. All samples must be thoroughly tested before being used.

Storage of blood in a refrigerator is carried out immediately since undue exposure to room temperature and unnecessary handling may result in hemolysis. The optimum storage temperature is 4° C. with a permissible range of temperature from 2° to 6° C. Citrated blood will freeze between 0 and –1° C. Attachment of the refrigerator to an electric circuit separate from that of all other electric appliances is advisable so that current failure will not so quickly occur.

If failure of refrigeration allows the temperature to rise, an alarm system and recording device alert the staff.

The blood is checked daily by the banker for evidence of hemolysis.

The equipment for procuring blood is essentially the same as that described for blood transfusion. Blood is dispensed daily during the regular banking hours except for emergency transfusions. Request is made to the lab-

Fig. 508. In a Red Cross Blood Center a nurse replaces her equipment after taking a pint of blood from a donor. A Red Cross volunteer nurse's aide lends assistance.

oratory to determine the patient's blood group, and the request, with the patient's blood, is sent to the bank, where cross-matching tests are made. All blood in the blood bank should have been previously grouped and subgrouped, including the Rh type.

Blood that has been removed from storage should be transfused immediately. It should be handled no more than necessary. An intern should remain with the transfused patient for at least the first 15 min. All reactions should be reported to the director of the blood bank service. All reports of transfusions should be returned to the blood bank within 12 hr. after transfusion.

It must be repeated here that all blood in the blood bank should be proved to have come from nonsyphilitic, nonmalarial, and nonhepatitis patients. Routine culture tests should be made to detect bacterial contamination.

No blood should be used that has been kept longer than 3 wk. Outdated bloods may be used to procure plasma, following the practice of removing plasma from the whole blood as outlined on p. 1589. In addition, the red cells may be saved for use in special medical and surgical conditions.

The American Red Cross program was instituted in this country to provide blood transfusion service for the smaller hospitals which cannot afford to conduct a blood bank of their own. There are now 45 Regional Centers.

AMERICAN RED CROSS BLOOD PROGRAM*

The American Red Cross had its first experience in blood procurement during World War II, when it collected approximately 14 million pints of blood which were turned over to commercial laboratories for processing for the Armed Forces. The Red Cross served only as a collection agency; all other processes were carried out either by the Army or Navy or by laboratories under contract to them. Following World War II, the Red Cross expected to leave the field of blood collecting and did. However, technical advances made during the war increased the demand for blood, particularly by physicians who had had available both blood and plasma and occasionally serum albumin. Because of this demand some Red Cross chapters were permitted to operate so-called "walking blood banks" on a community basis; others operated collection centers. Although these permissive programs served a need in limited areas, the need for blood was critical in many others. Upon the request of the Army and Navy, Veterans Administration, American Hospital Association, American Medical Association, United States Public Health Service, and other allied organiza-

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*This section has been prepared by the St. Louis Regional Blood Program, American Red Cross, St. Louis, Mo.

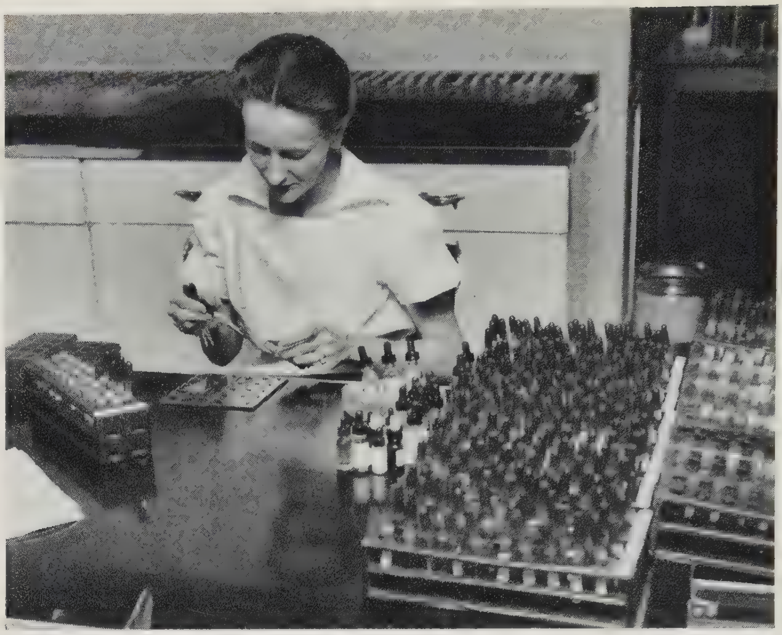

Fig. 509. Technician in the laboratory of the St. Louis Regional Blood Center making blood grouping tests, including the Rh factor. This is a mass operation. There were 410 blood specimens on the day the photograph was taken; each 2 represent a blood donor. In the background can be seen tubes lined up for the confirmatory tests using serum against known A and B cells.

tions, the Red Cross investigated the possibility of a national blood program.

With the endorsement in principle of its program, the Red Cross opened its first Regional Blood Center in Rochester, N. Y., in January, 1948. The St. Louis Center was the twelfth to open. Its opening date was August, 1948. There are now 55 Regional Centers. Fifteen Defense Centers came into being for the collection of plasma after the Korean conflict began.

A Regional Center opens in a particular metropolitan area after receiving approval of the American Hospital Association, Catholic Hospital Association, any hospitals not belonging to either, Medical Society, Public Health Service, and the Red Cross Chapter in that city and those in the surrounding areas expecting to be served by mobile units. Defense Centers opened with the same approvals plus an understanding that blood collected by these centers would not be distributed to hospitals in the area but would either be used as whole blood in Korea or processed by commercial laboratories as plasma, serum albumin, or immune globulin for the Armed Forces. Blood quotas of approximately 5% of the population per year were set for Regional Centers and about half that for Defense Centers.

The original aim of the Red Cross Program was to make blood and blood derivatives available to all whenever and wherever needed. It was realized by those outlining the program that this ambitious aim could be reached only through complete cooperation of the American public, which really is the American Red Cross. Cooperation had to come through many channels: (1) people must be donors so that the product would be available, (2) hospitals and doctors must be willing to use blood in a highly intelligent and conservative manner, and (3) technical personnel must be willing to learn enough about blood to be able to administer it with the least amount of hazard. Hospital administrative persons, technicians, nurses, and Red Cross personnel had to be able to explain the simple operating details of the program to recipients. These details are primarily concerned with why the administration charge is made and what it covers, why some hospitals continue to operate their own blood banks and receive little Red Cross blood, and why some hospitals receive their total supply from Red Cross, and other such questions.

The technical problems have been well handled by physicians and laboratory technicians. Those not previously accustomed to having blood available have sought and obtained the necessary information for safe administration. In explaining the technical aspects of the program, we must pay tribute to the outstanding experts in American medicine who outlined the technical operation of the program and who continue to give it direction. The official Medical Advisory Committee of the Red Cross Blood Program is the same as the Blood and Blood Derivatives Committee of the National Research Council. This committee participated in the writing of and approved the technical manual used by the various centers. The technical operation of each center has the approval of the National Institutes of Health and has a yearly inspection. The technical aspects of the program may be divided into 4 units for the sake of discussion: donor requirements and acceptance, collection technic, laboratory work, and distribution.

Donor requirements may be divided into those requirements set up for the protection of the donor and those requirements set up for the protection of the recipient.

Requirements protecting the donor

Age: Persons between the ages of 18 and 59 yr. are acceptable; persons from 18 through 21 yr. must have written parental permission unless married; persons on active duty in the Armed Forces are acceptable without parental consent.

Temperature and pulse: Temperature and pulse are checked, and any abnormalities are reported to the attending physician.

Weight: Donors must weigh at least 110 lbs.

Blood pressure: In general, persons with systolic pressure between 100 and 200 are acceptable. The physician checks on the medical history of some in this group and may reject them on his findings.

Hemoglobin: 12.5 gm./100 ml. for women and 13.5 gm. for men is considered the lowest level for acceptance. Those falling below this level are rejected by the physician. The hemoglobin test is made by the Phillips and Van Slyke copper sulfate specific gravity technic. A disposable lancet is used in pricking the finger. All lancets and needles used are of the disposable type in order to eliminate the danger of transmitting homologous serum jaundice from one donor to another.

History: In order that no one ill of some acute disease or chronic illness be taken as a donor, each donor is asked questions concerned with the following conditions: illness in past month, surgical operations in past 6 mo., tuberculosis, diabetes, rheumatic fever, cardiovascular disease, kidney disease, persistent cough, pain in chest, shortness of breath, fainting spells, and convulsions. Any person giving a positive answer to any of these questions is automatically referred to the attending physician who accepts or rejects him on an individual basis. Extreme apprehension is occasionally the cause for rejection. The physician makes an attempt to give the prospective donor a good understanding of why he is rejected, whether or not he may give blood at another time, and what course to follow.

Requirements protecting the recipient

Blood or plasma transfusion: No donor is accepted who has had a blood or plasma transfusion in the past 6 mo.

Malaria or antimalarial therapy: Donors who have had a history of malaria or who have received intensive suppressive therapy but who have had neither a clinical attack nor suppressive therapy in the preceding 2 yr. may be accepted for donations to be used as whole blood. Donors who have had neither a clinical attack of malaria nor intensive suppressive therapy in the past 6 mo. may be accepted for donations to be used as plasma or plasma fractions only.

Brucellosis: Persons having had undulant fever within the past 2 yr. are rejected. If the disease occurred more than 2 yr. ago, the donor is acceptable.

Jaundice: Persons having had infectious hepatitis or homologous serum jaundice within the past 2 yr. may not be donors. Persons having had contact with either disease in the past 6 mo. may not be donors. This blood shall be clearly marked for derivatives only.

The problem of contact with homologous serum jaundice and infectious hepatitis presents a real problem when considering laboratory technicians, nurses, physicians, and other hospital personnel as donors. There have been many reports of "needle jaundice" in persons performing venipunctures and examining the blood of patients. All laboratory personnel must exercise the greatest care to protect themselves from infection by the blood of any patient. Any break in the skin should be carefully protected.

Allergy, asthma, and hay fever: The transmission of nonspecific antibodies by stored blood does not present the great problem that it does in fresh blood; nevertheless, a person who feels well but gives a history of recent allergic manifestations is accepted for plasma or derivatives only.

Immunizations: In general, a person may give blood 24 hr. after receiving immunizations. There are exceptions, however.

Rabies: Donors may not be accepted until 1 yr. after the last immunization.

Smallpox: Donors may be accepted 2 wk. after an immune reaction or after the scab comes off.

Yellow fever: Donors may be accepted 2 wk. after the last injection.

Poliomyelitis: Donors may be accepted 24 hr. after the last injection, with the exception of oral immunization for poliomyelitis, in which case the donor would be accepted 6 wk. after the last immunization.

Typhoid, typhus, Rocky Mountain spotted fever, influenza, cholera, diphtheria, and tetanus: Donors may be accepted 24 hr. after the last injection.

Vitamin, hormone, liver, and other injections: Donors must be evaluated by the physician on an individual basis.

Cortisone therapy: Donors must be evaluated on an individual basis.

Collection methods

The actual collection technic depends to a degree on the type of equipment used. At the present time all venipuncture sets and bottles used are of the expendable type made by commercial houses. Whole blood collections are taken in bottles containing 110 ml. acid-citrate-dextrose (ACD) solutions.

The rubber stopper is prepared with 2% aqueous iodine and 70% alcohol before insertion of the needle. The arm preparation is made with 50% aqueous green soap and 2% aqueous iodine removed with 70% alcohol. Bleedings for ACD collections are performed in 5-7 min. Blood collected in ACD is carefully agitated approximately 5 times during withdrawal and is thoroughly shaken after withdrawal is completed. Immediate refrigeration is instituted.

We shall outline the procedures as carried out by the St. Louis Regional Blood Program. Blood collected in ACD solution is distributed only after the following laboratory work has been done:

1. Determinations of A-B-O groups by known antisera and known cells.
2. Determinations of the Rh factor, **Rh**$_0$ (D), **rh′** (C), **rh″** (E), and $\mathfrak{R}\mathbf{h}_0$ (D^u).
3. Serologic test for syphilis is the VDRL.
4. Titrations have been discontinued on all group O donors. Group O, ACD bottles are labeled "Isoagglutinin titer not determined." We discourage the use of group O blood as universal donor blood but realize that in cases of extreme emergency it must be used. Titrations should be carried out in hospital laboratories if group O blood is to be used as universal donor.

The A-B-O groupings and Rh typings are made by the slide method from clotted samples collected at the time of venipuncture. A Clay-Adams viewer is used for the Rh tests. Commercial sera of a potency meeting National Institutes of Health standards are used.

Although the standard 3-tube Kahn is used for the serologic test for syphilis in some Red Cross Blood Centers, many others are converting to the VDRL syphilis test. Donors with positive tests are followed up as recommended by the State Health Department.

The laboratory procedures are of the simplest type commensurate with the greatest accuracy possible. Perhaps the greatest problem for new personnel and a continuously troublesome problem for the experienced technician in mass typing is that of keeping all determinations related to one unit of blood carefully identified. The elimination of the human error in mixing samples and numbers relating to samples must be accomplished. In American Red Cross Blood Centers we have found the use of one number to identify a given sample and the use of one laboratory worksheet with no copying to be most helpful in preventing the most common errors in transcribing. "Reverse grouping" using unknown sera plus known A and B cells (2%) is made as a check against the original grouping tests. The value of 2 technicians reading the same test cannot be overemphasized.

Labeling, storage, and distribution of blood is the final unit for consideration. Every bottle is properly identified as to contents, including the formula of the anticoagulant, date of expiration (21 days from collection for ACD), name and address of collecting agency, and results of laboratory tests, i.e., serology, grouping, and typing. A whole blood number corresponding to that on the donor's medical history card appears on the bottle label and can be used to trace any unit of blood to the donor. Each ACD bottle has a pilot tube of clotted blood taken at time of venipuncture securely taped to it. This tube is taped to the bottle prior to the venipuncture and is not removed at any time in the Center.

Blood is stored at a temperature of 4°-6° C. in a refrigerator used for this purpose only and which has a constant recording device and alarm system.

The blood is distributed by the American Red Cross in precooled iced cases.

Chapter 81

Blood plasma

ADDINE G. ERSKINE

An enormous amount of literature has accumulated regarding the use of blood plasma as a substitute for whole blood for transfusion in certain indicated cases. The advantages of plasma are that it can be used without typing and can be stored in liquid, frozen, or dried states for months or years and then administered safely without significant deterioration of its component qualities. It is readily available for immediate use, and when injected, it does not increase the concentration of red blood cells in cases of shock and burns where hemoconcentration is already present. Manifestly, the use of whole blood where hemoconcentration is already present is contraindicated.

HISTORICAL DATA UPON THE USE OF LIQUID PLASMA*

Blood plasma as a substitute for whole blood was suggested as early as March, 1918, by Gordon R. Ward[309] in England. He pointed out that one of the chief troubles with whole blood is that the recipient's plasma might hemolyze the corpuscles of the donor. He made the observation that death from hemorrhage was not due to lack of hemoglobin but from loss of fluid, and thus the great need in these patients is for replacement of the depleted fluid. He suggested that this could be done by the administration of citrated plasma which could be preserved easily and injected safely.

Rous and Wilson[310] in 1918, while working on hemorrhage in animals, used plasma to replace the blood loss and pointed out that replacement of red blood cells is not essential because even in severe hemorrhage a sufficient number of cells remains to support life but that loss of blood volume is the important factor. They were able to restore the blood pressure to normal following gross hemorrhage and to maintain it by replacing the removed blood with an equal quantity of plasma. Where hemorrhage is too great, loss of red blood cells is important also.

Mann[311] in 1918 discussed the use of serum for treating surgical shock produced experimentally in dogs. He noted that parenteral injection of homologous serum produced results that were as good as or even better than any of the methods employed in the treatment of experimental shock. It is worthy of mention that his best results were obtained with relatively large doses of serum. He asserted that homologous serum might be of value under conditions in which serum could be kept and whole blood could not be obtained.

Strumia[312] began using human serum intravenously in 1927 for the treatment of severe infections. Later in 1927 he began using plasma because of the simplicity of preparation and its greater yield. It was noted then that even heterologous plasma given intravenously caused no reactions, whereas homologous sera commonly caused severe reactions. This difference in behavior of plasma and serum had previously been observed and commented on by Brodie[313] in 1900. Untoward reactions from serum were thought by him to be due to the process of fibrin precipitation when serum is separated from the clotting blood. This hypothesis continues to be conjectural.

Gradwohl[314] recommended plasma in the treatment of a severe burn case.

Weech et al.[315] in 1933, while performing plasmapheresis experiments in dogs, were able to produce a normal state in their animals by administering transfusions of normal dog serum.

The use of plasma intravenously in man as a hemostatic agent was reported by Filatov and Kartasevskij[316] in 1935. The same year Heinatz and Sokolow[317] used plasma in the treatment of hemolytic shock.

Elliott[318] in 1936 suggested the use of untyped serum and plasma for treatment of surgical, obstetric, or traumatic shock in which transfusions were indicated. He rationalized that the need for replacing the lost blood volume was more important than the red blood cells, since the maintenance of osmotic pressure is a function of the plasma proteins. Elliott also propounded the idea that liquid plasma could be stored for long periods without deterioration. The findings of other investigators have supported his contentions.

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*Many of these historical facts were found in the publication by Lt. Comdr. L. R. Newhouser, United States Navy, and Captain D. B. Kendrick, Jr., United States Army, Medical Corps, entitled "Human plasma" prepared April, 1941.

Fantus[319] in 1937 advocated the use of normal human serum because of its therapeutic and natural immunizing properties. He stated that in shock without hemorrhage and in burns intravenous injection of blood serum is much more rational than that of blood because these patients usually have an excess of red blood cells/c.mm.

Alovski and Burceva[320] published a favorable report in 1937 on the use of plasma as a substitute for whole blood in gynecologic conditions. Mahoney[321] in 1938 recorded encouraging results with preserved plasma in the treatment of experimental and clinical shock. Elkinton[322] and McClure[323] in 1939, working separately, obtained good results with plasma in burn cases. Tatum et al.[324] in 1939 suggested a technic for the preparation of whole blood substitutes in war conditions. Plasma was recommended as "an ideal substitute for whole blood in the emergency treatment of shock and hemorrhage from war wounds." They outlined the technic of collecting blood in a vacuum bottle, centrifuging the blood, and then aspirating the supernatant plasma into a second vacuum bottle for storage. In 1940 Elliott et al.[325] recommended the use of dilute, preserved, liquid plasma. The same year Strumia[312] advocated citrated blood plasma without cross matching for the treatment of burns and shock. Thalhimer[326] reported favorably on the use of convalescent serum for administering antibodies. Best and Solandt,[327] discussing their work on experimental shock, reported encouraging evidence in favor of using plasma and serum in preventing shock. Levinson et al.[328] have shown the value of serum in the treatment of posthemorrhagic shock in experimental animals. Kendrick[329] in 1941 advocated the use for military purposes of concentrated and normal dilutions of plasma for the prevention and treatment of shock in the combat zone. Kekwick and Whitby[330] in 1941, after using plasma for the treatment of shock and hemorrhage in air raid casualties in England, concluded that plasma and blood are equally effective in restoring blood volume in injuries of this type.

PREPARATION AND USE OF PLASMA

Plasma is the liquid portion of blood. In addition to fibrinogen, antihemophilic globulin, and prothrombin, all of which are involved in the clotting mechanism, plasma also contains antibodies present in the globulin fraction, crystalloids, electrolytes, and blood colloids, largely albumin, which exert osmotic forces for maintaining a normal blood volume. A supply of plasma ready for injection should be kept in blood banks for emergency use.

In order to obtain a maximum yield of plasma, whole blood is preferably taken in 4% sodium citrate. Fibrinogen is more stable when plasma is collected in sodium citrate than when ACD solution is used as the anticoagulant. Immediately after withdrawal, the blood should be placed in a refrigerator. When blood has been taken in sodium citrate, the plasma must be separated from the cells within 4 days of collection. When ACD solution is used, the plasma may be removed at any time within 26 days after storage in a refrigerator. See p. 1574 for plastic bags.

The blood may be centrifuged at 2000 r.p.m. for 1 hr. to separate the plasma from the cellular elements. If it is not centrifuged and the cells settle by gravity, the yield of plasma is reduced by approximately 15%. Centrifuged blood should be placed in a refrigerator overnight before the plasma is removed so that any cells that have been stirred up may resettle.

The plasma should be aspirated in a special room with no air currents, and all sterile precautions must be observed throughout its preparation.

Technic of preparation of plasma

This technic requires the use of equipment by which blood may be obtained and the plasma prepared under sterile conditions.*

It is necessary to have a supply of Transfuso Vacs of 500 ml. capacity. The bottle is a sterile vacuum unit designed to fit centrifuge cups. Sterile vacuum containers are available for removal of the supernatant plasma after centrifugation. These are known as Plasma Vacs, for the preparation of plasma. A Plexitron expendable set for the control of the blood flow into the sterile vacuum container is also necessary.

1. Prepare the blood donor in the usual manner. Cleanse the skin at the site of the needle insertion with alcohol-iodine. Constrict the upper arm with a short piece of rubber tubing or preferably with a blood pressure cuff.
2. Withdraw blood in the manner outlined on pp. 1572 and 1575. Before placing it in a refrigerator, break the vacuum by inserting a sterile air filter.
3. When the Transfuso Vacs are to be centrifuged, remove the air filter, place the bottles in centrifuge cups, and balance accurately. When centrifuging such large quantities, it is imperative that the bottles opposite one another be in perfect balance. If they are not, it is not possible to prepare clear plasma, and the centrifuge is likely to be damaged. For balancing, place rubber bands around the neck of the lighter Transfuso Vacs until they are in balance with the heavier ones.
4. Centrifuge for 45 min. to 1 hr. at 2000 r.p.m. to separate the plasma from the cells. Any jarring of the centrifuge or the Transfuso Vacs will disturb the cell layer, and poor plasma will result.

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*The equipment described here is manufactured by the Baxter Laboratories, Inc., Morton Grove, Ill.

5. For the removal of the supernatant plasma use an expendable aspirating set. Cut away the thin rubber disk.
6. Disinfect the stopper of the Transfuso Vac with alcohol-iodine and insert an air filter.
7. Remove the aspirating needle from its sterile wrappings and thrust it through the thin depression in the stopper into the plasma; then, resting both elbows on the bench, one on either side of the bottle, gently lower the aspirating needle into the plasma, controlling the action of the needle by holding the point against the glass wall of the bottle. Keep a bright light directly behind the blood bottle for ease in watching the needle and the plasma level. It is important that the needle of the air filter be above the surface of the plasma. After inserting the long aspirating needle, open the clamp gently without disturbing the bottle and allow the plasma to flow into the Plasma Vac. Take care that no air is sucked into the plasma bottle. See Fig. 510.
8. The cellular elements in the blood bottle must not be disturbed. If cells are inadvertently drawn over with the plasma, the plasma bottle must be recentrifuged and the supernatant plasma removed from the cells through a closed system.
9. After all the plasma has been removed, close the clamp and allow the small amount of plasma remaining in the tubing to run into sterile test tubes by holding the tubing vertically and opening the valve slightly. Then test the plasma samples for sterility by anaerobic and aerobic cultures. If the plasma is found to be sterile after sufficient time has elapsed, it is then ready for use or dilution. Do not use plasma containing undue amounts of fats or any that show signs of high bilirubin content.

The completed pooled plasma or serum in accordance with the regulations of the National Institutes of Health relating to plasma shall contain not more than 25 mg. of hemoglobin/100 ml.

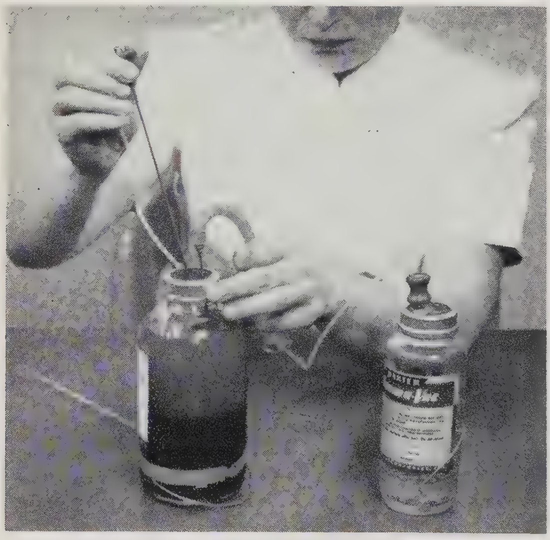

Fig. 510. Aspirating plasma. The plasma will be drawn off into a vacuum container. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

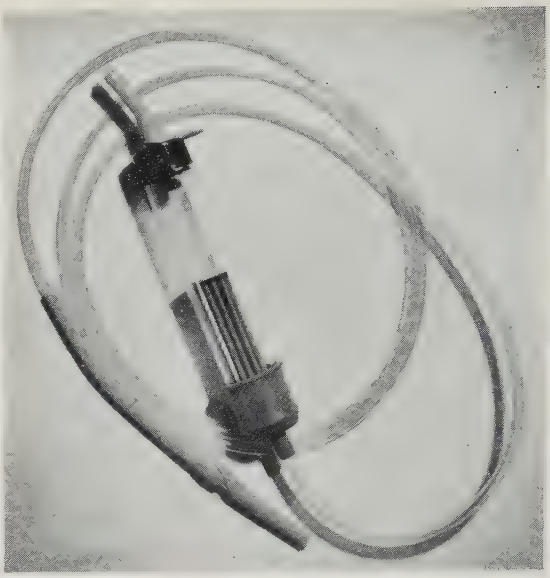

Fig. 511. Sterile expendable Plexitron set for administering blood, plasma, or serum. (Courtesy Baxter Laboratories, Inc., Morton Grove, Ill.)

If the cells remaining in the blood bottle after removal of the plasma are to be kept for cell suspension transfusion, close the hole in the blood bottle stopper aseptically by encasing the top of the bottle with a tight-fitting sterile hood. A different aspirating needle must be used in each blood bottle when the cells are to be used for transfusion.

When all the plasma has been aspirated from one bottle of blood, close the clamp and withdraw the aspirating needle from the blood bottle. Using all sterile precautions, enter another blood bottle first with the airway and then with the aspirating needle; then release the clamp. The needle in the pooling bottle remains in place. Continue until the desired amount of pooled plasma is obtained.

The isoagglutinin titer of pooled plasma is less than that of separate plasma, not only because the dilution lowers the titers but also because groups A and AB plasma contain some group A-specific substance and groups B and AB plasma contain some group B-specific substance, which will partially neutralize their respective isoagglutinins. A 2 L. pool of plasma should contain not more than 3 units of group O plasma, 2-4 units of group A, and 1-3 units of group B or AB plasma. When plasma is stored at room temperature after pooling, the isoagglutinin titer decreases further.

Withdraw 10 ml. for each 1000 ml. plasma from the pooled plasma, using a glycerinated Luer-Lok syringe and needle, and eject into fluid thiogly-

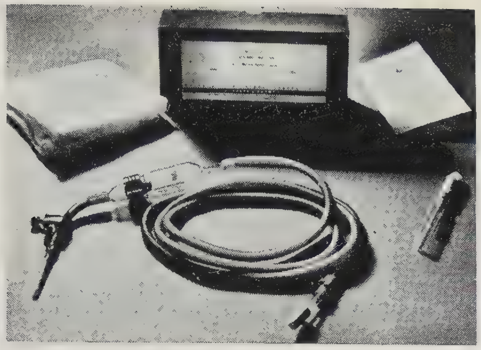

Fig. 512

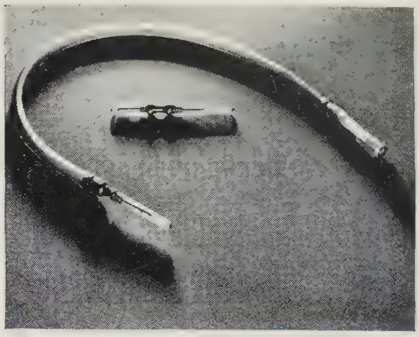

Fig. 513

Fig. 512. Lyovac normal human plasma and equipment. (From 'Lyovac' normal human plasma, West Point, Pa., 1941, Merck Sharp & Dohme.)

Fig. 513. Administration set for reconstituted plasma. (From 'Lyovac' normal human plasma, West Point, Pa., 1941, Merck Sharp & Dohme.)

collate medium. Incubate the culture at 30°-32° C. for 2 wk. as a sterility control. When sterility has been proved, divide the pooled plasma into several smaller containers, each of approximately 300 ml. size, using the same technic as in the pooling process. Make cultures from the pilot sample taken at the time of division.

Label each bottle clearly, indicating the expiration date. Liquid plasma has an expiration date of 2 yr. from the time of withdrawal of the oldest blood in the pool. It should be stored at slightly higher than room temperature.

Homologous serum hepatitis may be transmitted by whole blood, plasma, serum, thrombin, or fibrinogen. The greater the size of the pool of plasma, the greater the number of cases of hepatitis in the event the pool contains virus. Limiting the pool size to less than 10 blood donations and storage of liquid plasma at 30°-32° C. for 6 mo. or longer greatly reduces the risk of hepatitis. Evidence indicates that shorter time at higher temperature or longer time at lower temperature and the use of ultraviolet irradiation with a shorter storage period in the liquid state may be equally satisfactory.

Plasma from the blood of a single donor may be aspirated into another container and administered to a patient from this container. This is called "single donor plasma." If it is not intended for the treatment of coagulation defects, it may be stored at room temperature.

Fresh frozen plasma

Factors that correct the clotting defect in hemophilia are thermolabile and disappear quite rapidly from plasma stored at room temperature, but they are retained in fresh plasma and in plasma frozen shortly after collection. Fresh frozen plasma is usually prepared from a single donation and not from a pool.

Special attention must be taken to maintain a low temperature during processing and storage of the plasma. The plasma must be frozen within 4 hr. of bleeding the donor and at −18° C. or colder. It must be thawed rapidly just prior to administration. When stored at this temperature, the antihemophilic activity remains relatively unchanged for months, perhaps years.

1. To prepare fresh frozen plasma, centrifuge the blood at 2800 r.p.m. for 10 min. as soon as possible after collection (the total centrifuge time is approximately 40 min. from beginning to end since it requires some time to reach the proper centrifuge speed and to permit the centrifuge head to coast to a stop). Pre-chill the centrifuge cups in a refrigerator.
2. Use ice water to balance the centrifuge cups.
3. Pre-chill the flasks that are to receive the plasma.
4. Take a sample for culture at the time the plasma is aspirated.
5. Attach a pilot sample of plasma securely to the bottle for cross matching and to check on a possible reaction. If a group AB donor is used, no pilot sample is considered necessary in some banks, but this is not recommended. Some blood banks use only group AB blood for preparing fresh frozen plasma.

The quantity of plasma in each container may be varied: 50 ml. for an infant, 125 ml. for a child, and 250 ml. for an adult.

6. As soon as the bottle is filled and labeled, place in a freezer in a slanting position next to the wall containing the coils. After freezing, place the bottle in an upright position. If the plasma thaws during storage, it must be discarded.
7. Transport the bottle in dry ice outside the blood bank.
8. To prepare the plasma for injection, thaw in a 37° C. water bath or a pan of tepid water and administer **immediately** after thawing. **Discard if not used.**

Plastic bags may be used for this process. When using plastic bags, a heavy object should be placed on the bag during the freezing process to make a dimple, which will disappear if the plasma thaws without the knowledge of the bank personnel.

Plastics that have been subjected to low temperatures become brittle and may crack or break easily, so that plastic bags containing frozen plasma should be handled with care. Upon removal from a freezer, a plastic bag should remain at room temperature for 5-10 min. before being placed in a 37° C. water bath.

PLASMA AND SERUM FRACTIONS*

Normal human serum albumin: Normal serum albumin, human, is usually distributed in a 25% salt-poor solution in three sizes: 20, 50, and 100 ml. The 100 ml. unit is osmotically equivalent to 500 ml. citrated plasma.

Albumin is used in the treatment of shock, burns, and hypoproteinemia. It is free of the homologous serum hepatitis virus and for this reason is preferred to plasma. However, for the preparation of one 100 ml. unit of normal serum albumin, 3.6 donations of blood are required, which makes the cost relatively high and therefore limits its use.

When whole blood and plasma are judiciously administered, they are usually as effective as or more effective than albumin in diseases in which albumin may be used.

Fibrinogen: Fibrinogen is now commercially available. It is used in any condition associated with a fibrinogen deficiency. Such a deficiency is suspected if blood does not clot or if clotted blood reassumes the liquid state. Fibrinogen deficiency is rare. When present it may result in extensive hemorrhage. It is an infrequent complication of childbirth and chest or abdominal surgery. There is also a congenital form of fibrinogen deficiency. Refer to p. 1227.

Fibrinogen is available in dried form in 1 gm. quantities. It is dissolved in water and administered intravenously. The purified product is more useful than fresh blood in controlling hemorrhage, especially in obstetric hemorrhage due to afibrinogenemia or fibrinolysis. There is great risk of transmitting hepatitis by the use of purified fibrinogen and therefore it should be used only in emergency. A pint of fresh blood may contain 0.5-1.5 gm. fibrinogen.

Antihemophilic globulin: Antihemophilic globulin is not commercially available as yet (1963), but it has been prepared in dried form. It must be reconstituted with water for intravenous administration. It is prepared from fresh blood during the fractionating process. The quantity to be injected into a hemophiliac depends on the ability of the given lot to correct the clotting defect of the individual patient.

Specific antibody immune serum (human convalescent serum)—measles, mumps, pertussis, poliomyelitis, and scarlet fever immune sera: Immune sera from convalescent donors or immune globulins are used for passive immunization against certain infections. These sera have high antibody content and confer a short passive immunity, from 3-5 wk. The immunity is short lived because the antibody proteins are metabolized and excreted. Such therapy is more useful for prophylaxis than for treatment of established disease. No standard of potency has been established for most of these sera except in the case of poliomyelitis serum. Pertussis immune serum can be standardized, but this is not required. There is danger of transmitting homologous serum hepatitis by the use of immune sera. Effective antibiotics and sufficient quantities of immune globulin are used more often than the immune sera.

Human convalescent sera for the above diseases are prepared by pooling the serum from adult donors not more than 6 mo. after convalescence from the specific infectious disease. In poliomyelitis, donors may be accepted from 2-5 yr. after recovery from an attack of paralytic poliomyelitis. Pertussis serum comes from donors who have had whooping cough, and they are given pertussis vaccine prior to blood donation. Manifestly, donors must be selected with great care. The pooled serum is filtered through a Berkefeld N filter and radiated with ultraviolet light.

Merthiolate is used as a preservative in the liquid sera. Sterility and toxicity tests are performed. The expiration date of liquid sera is 1 yr. from the date of issue. The date of issue may be preceded by a 2 yr. storage period at a temperature not exceeding 5° C.

Immune sera are usually administered intramuscularly, but they may be given intravenously if the product has aged for 1 mo. Dosage schedules are recommended by the manufacturer.

Immune globulin for measles and poliomyelitis: The gamma globulin fraction of plasma usually contains the highest antibody concentration. The gamma globulins comprise about 16% of the total plasma proteins.

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*The section on plasma fractions has been abstracted from Chapter VI of Technical methods and procedures, revised edition, 1960, by permission of the American Association of Blood Banks, Chicago, Ill.

Citrated plasma, plasma salvaged from blood taken in ACD solution, reconstituted lyophilized plasma, and blood collected from placental sources may all be used for fractionation. Each lot of finished product represents a plasma pool from more than 1000 donors. The final product is a 16.5% ± 1.5% solution of globulin in a 2.25% solution of glycine.

Immune serum globulin is effective in prophylaxis of measles, poliomyelitis, and infectious hepatitis but not of homologous serum hepatitis.

It has also been used advantageously in maintaining general resistance against infections in patients with agammaglobulinemia, hereditary or acquired. It is administered only intramuscularly.

PLATELET TRANSFUSIONS

At times it is necessary to transfuse blood platelets to correct a platelet deficiency. This may be accomplished by the use of platelet-rich whole blood, platelet-rich plasma, or platelet suspensions. As many intact platelets as possible should be retained.

The blood must be exposed only to nonwetting surfaces during collection, storage, and administration. Plastic apparatus or apparatus coated with silicone or other similar agents may be used. The transfusion must take place as soon after blood donation as possible, preferably within 4 hr. Platelets that have been stored for 24-48 hr. have a shorter survival time than do fresh platelets, but they are still effective in the treatment of platelet deficiencies. The blood should be taken from donors whose blood is rich in platelets, and although blood from normal donors does not have as high a platelet count as blood from, for example, a polycythemia patient, it often will help correct a platelet deficiency.

Platelet-rich plasma is prepared by aspirating plasma from several units of blood that have been centrifuged slowly, collecting the plasma in a pooling bottle. The platelet suspensions are prepared from this plasma pool by centrifuging it hard and then removing the clear plasma, retaining the platelets. A more or less direct transfusion from donor to recipient, using siliconed needles and syringes, may also be used to transfer platelets from one person to another.

A method of separation and storage of platelets and at the same time obtaining plasma rich in antihemophilic globulin and packed red cells has been described by Strumia.[331] The whole blood is taken from normal, healthy, fasting donors or from polycythemia patients who are bled for therapeutic reasons. These individuals are preferably group O. Collect the blood in 500 ml. plastic bags containing the standard amount of ACD solution. All tubing and collection receptacles should be plastic and the needles must be carefully siliconized.

1. Mix the blood rapidly and with minimal agitation, and carry out the same precautions in subsequent handling. Cool the plastic bags containing the blood promptly; then centrifuge in a cold room or pack the bags in ice and centrifuge at less than 500 g for 20 min., which will sediment the red cells.
2. Transfer the supernatant platelet-rich plasma through a closed system, using plastic tubing and siliconized needles, to an empty 300 ml. plastic bag without disturbing the erythrocyte layer. In making the transfer gently press the bag containing the centrifuged blood, beginning at the lower part of the bag and proceeding gradually to the upper part.
3. Centrifuge the separated platelet-rich plasma in the cold for 1 hr. at 2000 r.p.m. with a centrifugal force of 1800 g or more. The platelets and leukocytes will be entirely sedimented in the form of a white, viscid material.
4. Remove the supernatant platelet-free plasma and transfer to a sterile plastic bag or glass bottle, leaving 25-40 ml. plasma remaining in the bag.

The platelet-free plasma is rich in antihemophilic globulin. It should be shell-frozen immediately and freeze-dried as soon as possible for optimal preservation. Plasma properly prepared and freeze-dried maintains its essential properties for approximately 5 yr.

Stir up the platelet concentrate by gentle agitation in the bag. Remove a small sample and test for sterility and also make a count of the platelets. Freeze the bags with the concentrated platelets by using ethanol-dry ice mixture and maintain in a frozen state at −20° C. or lower. The platelets should also be tested for thromboplastic activity by means of the thromboplastin generation test.

Transfusion of concentrated platelet suspensions may cause vasospasm if carried out too rapidly, but the platelets that correspond to the content of 500 ml. blood may be safely transfused over a period of about 15 min.

Strumia has stated that whole blood collected in plastic apparatus and immediately cooled and stored at 1°-2° C. retains active platelets for at least 74 hr.

PYROGENS

In the preparation of accessories all distilled water should be pyrogen-free, sterile, and otherwise suitable. What is meant by **pyrogen-free** distilled water? In 1911 Hort and Penfold[332] showed that the intravenous injection of freshly distilled water caused no symptoms in animals and in man, whereas the intravenous injection of the same water, after it had been incubated in unsterile vessels, caused a febrile reaction. These writers decided that this principle, provisionally called **pyrogen**, was a product associated with bacteria but was not a part of the bacterial bodies themselves. The fever-producing principle was still present after the water had been passed through a Berkefeld filter. These facts were confirmed by Siebert[333] in 1923 by injecting distilled water intravenously into rabbits. In 1934 Banks[334] reported that *Pseudomonas urea* and *scissa,* which he isolated from surface waters, were pyrogenic. Most of the work on pyrogenic substance has been confined to the bacteria found in waters. Co Tui and Schrift[335] showed that pyrogen production is much more widespread than was first embraced in the concepts of Siebert and Banks. They investigated 18 strains of microorganisms of different genera, tribes, and families, making tests (1) with bacterial strains grown in initially pyrogen-free broths; (2) using bacteria that were most convenient to grow on slants or in Kolle flasks, washing out the colonies with pyrogen-free physiologic saline containing 0.4% phenol, inactivating for 45 min. by heat, then filtering and testing the sterile filtrate; (3) testing 9 organisms in the form of vaccine made with pyrogen-free water. All the vaccines were passed through a Berkefeld filter.

Their estimate of a pyrogenic reaction in a dog was (1) a leukopenia 30-45 min. after the intravenous injection of the pyrogenic material and (2) a rise in temperature 30 min. after injection, reaching a peak in 2-4 hr. and returning to normal in 4-5 hr. In mildly severe reactions there were prostration and gastrointestinal symptoms, such as vomiting and diarrhea. The vomitus and stool may contain blood. These symptoms are comparable to those in man.

Pyrogenicity of bacterial contaminants found in biologic products

Probey and Pittman[336] investigated the pyrogenic properties of those microorganisms isolated from blood or plasma during the various stages of processing dried normal human plasma, to determine the quantitative and qualitative nature of the thermal response of rabbits (1) to living bacteria, (2) to heat-killed bacteria, and (3) to whole cultures grown in serum.

The materials used were 49 cultures from 8 processing laboratories widely distributed throughout the United States. Twenty-eight representative cultures were selected for study including staphylococci, micrococci, streptococci, gram-negative bacilli, and spore-forming and nonspore-forming gram-positive bacilli. The sodium chloride solution, which was used as the diluent, was prepared with one lot of sodium chloride proved by animal test to be pyrogen-free and with water freshly redistilled in a glass still. Each lot was tested and accepted for use only if the rabbit thermal response induced was characteristic of pyrogen-free saline.

Methods

The thermogenic properties of the test material were determined by the pyrogen test as described in the *U. S. Pharmacopoeia* XII, with a few changes.

The pyrexial response induced by the bacterial suspensions was computed by 3 procedures: (1) **high above normal,** the U.S.P. method, bases the computation on the temperature deviation from the pretreatment normal temperature; (2) **high above control** records the difference between the maximum temperature deviation induced by the test material and that of the pyrogen-free control; and (3) **peak above control** computes the pyrexia as the difference between the temperature induced by the test solution at the time of maximum temperature rise and the temperature of the pyrogen-free controls at the corresponding hour.

Since the pyrogen-free saline control in practically every instance induced a depression from the preinjection normal temperature, the normal physiologic reaction of the rabbit to injection of pyrogen-free saline is depression of the normal temperature. For this reason Hort and Penfold believed that the evaluation of the pyrogen test should be predicated upon the normal physiologic response of the rabbit to injection with a pyrogen-free control and not upon the pretreatment normal temperature as is now recommended by the *U. S. Pharmacopoeia.* Another observation is that the quantitative characteristic of pyrogens, as reported for the bacteria studied, indicates that a slight febrile response in rabbits, of less than the 0.6° C. temperature elevation permitted by the U.S.P. XII test, is in fact due to pyrogen contamination. This marginal amount of pyrogen may be sufficient to cause clinical reactions in human beings, since Co Tui and Schrift have observed that the rabbit is only ⅓ as sensitive to pyrogens as is man. Co Tui[337] later recommended that the official test be more quantitative and suggested the use of several test doses to ensure freedom from marginal pyrogen contamination. Until the characteristics of pyrogens are more fully elucidated, the observation period of the animal thermal test should be continued until the maximum response has been reached or until the temperature curve has definitely entered the downward phase. Work on contaminated blood or blood plasma during the processing of dried normal human plasma indicates that the contaminating bacteria are all capable of inducing fever in rabbits and that the count of bacteria in a contaminated blood product does not furnish an index of pyrogenicity.

The dog has a much more stable thermoregulatory mechanism but is less sensitive to pyrogen than is the rabbit. A positive test in the dog is so characteristic, however, with the additional symptoms of leukopenia, vomiting, and diarrhea, that it is unmistakable. For this reason a positive test in the dog is much more significant than a negative one. To summarize, the rabbit is the better animal to test for the absence of pyrogen, whereas the dog is the better animal to establish the presence of pyrogen. Both animals must be used to complete the picture. Both dogs and rabbits have been used for several positive pyrogen tests without developing manifest immunity or tolerance.

The rabbit is approximately 3 times, and the dog at least 6 times, less sensitive than man to purified pyrogen from typhoid vaccine.

Test dosages: Assuming that the above figures hold for pyrogen from sources other than the typhoid bacillus, the rabbit should receive as much as 3 times the maximum per kg. dose in man and the dog 6 times. In order to approximate the dose in man, the rabbit test dose must therefore be not less than 50-100 ml./kg. body weight. Although this amount of fluid almost approximates the total blood volume of the animal, if the dose is injected in 10-30 min., the rabbit can easily take care of the plethora. But, in any event, the symptoms of plethora are different from those of a pyrogenic reaction. Since the dog is to be used as a **positive** test animal, any dose that elicits the reaction shows the degree of pyrogenicity of the solution. This can be arrived at by trial and error, starting from a minimum dose of 250 ml.

Procedure of the test: The test is run in 2 parts, the preliminary part on rabbits, the confirmatory part on dogs.

Preliminary test: Use albino rabbits of 2½-3 kg. These rabbits should previously have been conditioned for several days to handling and taking of rectal temperatures until these procedures provoke no struggling and their rectal temperature readings are constant within 0.25° C. in 3 readings taken 15 min. apart. Before injection, temperature readings taken at 15 min. intervals must not be over 39° C. The thermometers must be inserted the same distance beyond the internal sphincter of the rectum and allowed to remain there for 2 min.

Warm the test solution to nearly the temperature of the animals; inject 50 ml./kg. body weight into one animal and 100 ml./kg. body weight into the second animal no faster than 10 ml./min. Take the temperature readings every half hour for 4 hr. after the injection. A rise of over 5° C. in 1-3 hr. after injection is taken as presumptive evidence of the presence of pyrogen. A pyrogenic sample ought to give a more severe response with 100 ml. than with 50 ml.

Confirmatory test: This is not necessary where a positive presumptive test is enough to lead to the condemnation of a product as unfit for human use. When more positive evidence may be required in many legal or medicolegal complications, the clear-cut result of a positive dog test is not easy to controvert. Perform the test as follows: One healthy dog of 15 kg. body weight, conditioned to the taking of rectal temperature, with an initial rectal temperature of not over 39° C. and blood count not exceeding 20,000 white blood cells/cu.mm., is used. Depending upon the response of the rabbits, give 250-1500 ml. of the fluid intravenously through one of the external jugular veins, not faster than 30 ml./min. Take the blood count again 45 min. and the temperature every 30 min. after the injection. Leukopenia, a fall of 5000 cells/cu.mm. or over and temperature rise of 5° C. from 1-3 hr. after the injection are evidences of the presence of pyrogen. The occurrence of emesis, prostration, and diarrhea are added checks.

Pyrogen production is more common than is generally believed. It is not confined to any one group but occurs in a number of different groups of bacteria. Nor is it associated with nucleic acid, with pathogenicity, chromogenicity, or with the property of taking the Gram stain. The production of pyrogen by a member of the yeast group suggests that even higher forms than bacteria may possess this property of pyrogen production. Chemically and immunologically, pyrogen is still an unknown entity.

The importance of pyrogenic substances resides in the fact that they cause reactions from the use of improperly cleaned infusion equipment and intravenous solutions, such as Ringer's solution, glucose solution, citrated solutions of defibrinated blood, etc.

DRIED PLASMA*

In order to provide plasma in a compact form, resort has been made to the use of the lyophil technic, such as was worked out by Flosdorf and Mudd.[338] These two workers determined that the content of antibodies and complement in serum rapidly frozen and then dehydrated in the frozen state under high vacuum shows no detectable decrease in the processing and, furthermore, that the rate of the subsequent deterioration is reduced to a small fraction of that which takes place in the liquid state. Scudder[339] stated that whole blood deteriorates rapidly, whereas lyophilized plasma may show no appreciable change even after years. By the electrophoretic method of analysis the protein patterns for the lyophilized plasma differ little from that of the fresh control, whereas protein patterns dried at 37° C. show wide variation.

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*Liquid plasma is preferred. Drying plasma preserves the hepatitis virus. Liquid plasma kept at room temperature for 6 mo. or longer loses its capacity to cause homologous serum jaundice.

A number of methods have been described, but the recommendation is made that if one desires to use dried plasma, it should be purchased from a reliable firm.

One finished lyophilized product is known as Lyovac* Normal Human Plasma. When desiccated, it retains its therapeutic value and remains stable for at least 5 yr. and is available for use by the addition of pyrogen-free distilled water. It is stated that the content of specific and nonspecific antibodies, complement, and coagulating elements, together with ⅗ of the platelets, is essentially the same as that of the original fresh plasma.

Precautions regarding the use of lyophilized plasma:

1. Remove the needle before all the water runs in.
2. Do not warm.
3. Dissolve in 5 min., shaking at room temperature, to obtain a clear solution.

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*Sharp & Dohme, Philadelphia, Pa.

Chapter 82

Medicolegal aspects of blood grouping

ADDINE G. ERSKINE

It must be accepted that once the blood group of an individual is established it remains unchanged throughout life. This basic fact constitutes the principle upon which forensic examinations of blood are made. Today the evidence of blood tests is routinely accepted in many courts, and many states have passed statutes empowering their courts to order such tests whenever parentage is disputed. New York, in 1935, was the first state to adopt a blood test law. From March 22, 1935, until November 17, 1949, almost 15 yr., the Court of Special Sessions in New York City had never adjudged as a father of a child a man excluded by blood tests. This record was broken, however, on November 17, 1949, by the court's decision in a case in which the accused was declared the father of a child despite the fact that 2 independently performed blood tests excluded him as a possible parent.[340] As Wiener[341] has observed, the reliability of the blood grouping test is still so great that no other type of evidence can approach it. The Institute of Forensic Medicine of Copenhagen and the Danish Medicolegal Council have submitted to the Danish courts written instructions regarding the reliability and judicial acceptance of blood test exclusions, concluding as follows[342]:

1. Paternity incompatible with the A-B-O system can be excluded with just as great a certainty as may be given by any biologic method.
2. Paternity incompatible with the M-N system can be excluded with just as great a certainty as may be given by any biologic methods.

It is emphasized that no deviation from the inheritance of the M-N system has ever been seen. (Refer to factor **M**^{e}, p. 1551.)

Likewise, the possibility of an erroneous exclusion of paternity after the A-B-O system may also be disregarded in practice.

The Danish courts are thus advised by their Medicolegal Institute that the blood test exclusions are entirely reliable and should be accorded decisive effect. It must be noted that these tests must be performed by experts especially trained for this examination.

Blood tests are also used to solve other legal problems, notably in assisting to determine whether blood stains have come from one person or another, particularly in cases of murder, rape, and hit-and-run driving. There are certain limitations to the use of blood grouping in determining innocence or guilt when examining blood stains. Since the majority of persons belong to either group O or group A and only a smaller percentage to groups B and AB, the tendency of the tests might more frequently suggest innocence rather than guilt. For example, the blood composing a stain on a suspect's clothing and that of the true criminal may be of the same group. A defense could be raised that the stains upon the clothing of the accused were produced by his own blood. The value of the test is of course apparent when the blood stains under examination are of the same group as the blood of the victim but of a different group from that of the suspect. Blood grouping reactions reduce the given number of individuals from whom a given sample of blood may have been derived.

BLOOD GROUPS AND HEREDITY

With the present knowledge of genetics it has become evident that problems of disputed parentage can be clarified by the application of the mendelian laws of heredity to the problem of blood groups. Fortunately, the blood groups are inherited precisely by a relatively simple mechanism. It must be emphasized that blood tests can be used only as negative proof of parentage, i.e., they can **disprove** paternity or maternity but they cannot **prove** them.

The serologic differences in the blood cells have been proved to be determined by heredity and are not influenced by environment. Ottenberg and Epstein[343] and von Dungern and Hirszfeld[344] proved that the 4 Landsteiner blood groups are inherited. Bernstein[345] in 1925 established the exact mechanism of heredity. These inherited factors follow Mendel's laws. With respect to what is meant by mendelian dominants, one must turn to the painstaking research of Johann Mendel, born in 1822 of German parentage in the village of Heinzendorf in Silesia. At the age of 21, in 1843, he entered the Augustinian Monastery at Brünn, Austria, there spending the remainder of his life except for a short period of study at Vienna. His name upon entrance to the Monastery became Gregor, and now he is known as Gregor Johann Mendel. In a little strip of garden behind the monastery building, Mendel conducted his experiments upon hybridization. In his rooms he hybridized mice, along the edge of the garden he experimented with bees, and in the garden itself, he crossed many kinds of plants. His famous experimental work was performed with garden peas, columbine, snapdragons, slipperworts, pumpkins, four-o'clocks, beans, etc. His work with garden peas is the most notable. Seven pairs of peas with contrasting characteristics were used by him, including smooth seeds and wrinkled seeds, yellow cotyledons and green cotyledons, inflated pods and constricted pods, yellow pods when unripe and green pods when unripe, flowers in the axils of the leaves and flowers at the ends of the stems, white seed coats and brown seed coats, tall plants and dwarf plants.

Mendel crossed tall plants with dwarf plants and produced the first filial generation, known as F_1, all of which were tall. When these tall plants were selfed, they produced both tall and dwarf plants, there being 3 times as many tall plants as dwarf plants. In the second generation ⅔ of the tall plants when selfed produced tall and dwarf plants in the ratio of 3:1, just like the F_1 plants. All the F_1 plants were tall, because, according to Mendel, the factor determining tallness was **dominant** to the factor for dwarfness. When hybrid tall plants were selfed, tallness showed up in the proportion of 3:1. The factor that did not produce its effect in the presence of the contrasting factor he called **recessive.** Thus, **tallness** was the dominant factor and **dwarfness** was the recessive.

Mendel's work was published in 1866, but very little attention was paid to it until the botanists deVries, Correns, and von Tschermak reached conclusions similar to his but independent of each other. His paper was then rediscovered and credit given to him after 30 yr. Thus in 1900 his work was made known to the scientific world.

Classifications based upon genetic formulas are known as **genotypes,** whereas the final observable results are known as the **phenotype** (refer to pp. 1492 and 1493). Thus for phenotype A the possible genotypes are *AA* and *AO*, whereas in group B (phenotype B), *BB* and *BO* are the possible genotypes corresponding to phenotype B; in group O the genotype is *OO*, the phenotype O; whereas in group AB the genotype is *AB*, and the phenotype is likewise AB. The various genotypes have been worked out by experts, using serologic methods and family studies, and these form the basis of study in heredity of blood groups.

The units of heredity occur in pairs, one member of each pair being contributed by the father and the other by the mother. When the egg or sperm cells are produced, the members of each gene pair separate so that each cell contains only a single gene of each kind. When the sperm fertilizes the egg, the **zygote** is formed, and the genes are paired again in the offspring.

Agglutinogen M is determined by the gene *M* and agglutinogen N by the gene *N*, so that only 3 combinations of the genes in the M-N system are possible: *MM*, *NN*, and *MN*, corresponding to 3 blood types, M, N, and MN. When an individual of type MN is

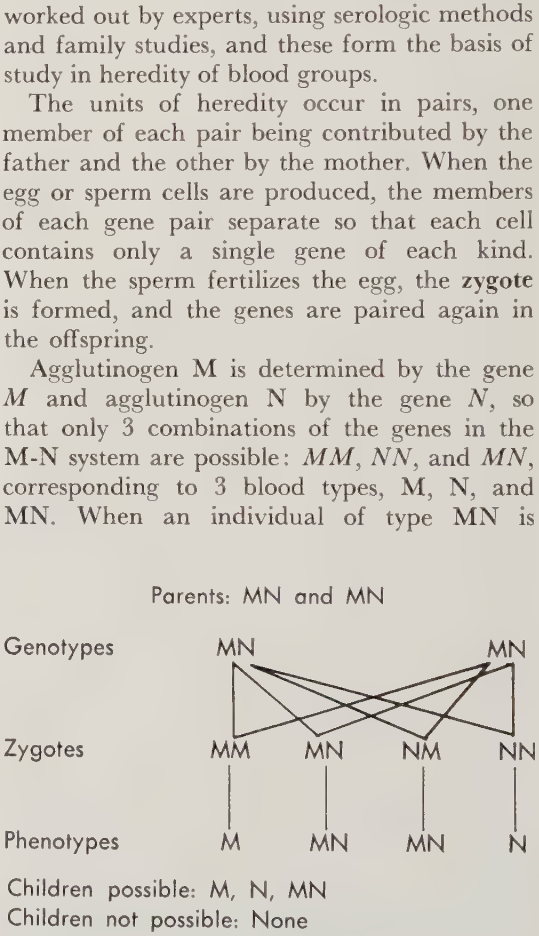


Fig. 514. Mating MN × MN.

	Father		Mother
Parents' phenotypes	Rh_1rh		Rh_2rh
Possible genotypes	R^1r	×	R^2r
	R^1R°	×	R^2R°
	$R^{\circ}r'$	×	$R^{\circ}r''$

		Sperm			
		R^1	R°	r'	r
Ova	R^2	R^1R^2	R^2R°	R^2r'	R^2r
	R°	R^1R°	$R^{\circ}R^{\circ}$	$R^{\circ}r'$	$R^{\circ}r$
	r''	R^1r''	$R^{\circ}r''$	$r'r''$	$r''r$
	r	R^1r	$R^{\circ}r$	$r'r$	rr

Possible genotype in children, rearranged:

rr; $r'r$; $r''r$; $r'r''$; $R^{\circ}R^{\circ}$ and $R^{\circ}r$; R^1r, R^1R°, and $R^{\circ}r'$; R^2r, R^2R°, and $R^{\circ}r''$; R^1R^2, R^1r'', and R^2r'.

Possible phenotypes in children—all others excluded:

rh, rh′rh, rh″rh, rh_yrh, Rh_o, Rh_1rh, Rh_2rh, and Rh_zRh_o.

Fig. 515. Method of working out a sample mating to determine possible phenotypes of children in the Rh-Hr system. (Courtesy Dr. A. S. Wiener.)

mated to another individual of type MN, the children possible to this mating are shown in Fig. 514.

Because of the large number of blood factors and hence the various possible phenotypes in the Rh-Hr system, the determination of children possible to certain matings is more complex. Fig. 515 is an example of a method of determining the possible phenotypes of children in matings in the Rh-Hr system. Wiener has selected as an example a father of type Rh_1, phenotype Rh_1rh, and a mother of type Rh_2, phenotype Rh_2rh. (The determination of the phenotypes and the possible genotypes is given in full in Table 314.) The children possible to such a mating are given in Fig. 515. See also pp. 1607-1612.

BLOOD GROUPING TESTS IN THE EXCLUSION OF PATERNITY

In order to gain a complete understanding of this subject, the monograph by Wiener and Wexler, *Heredity of the blood groups,*[346] should be thoroughly studied. Because the human blood group factors have been shown to be inherited, blood grouping tests can be applied to the problem of paternity. Wiener has outlined types of cases in which such tests are applied. Unger has also reported important cases and observations on this subject.[347]

1. A man accused of the paternity of a child born out of wedlock denies the charge. This is the most common type of case for which blood grouping tests are ordered.
2. A man accused of assault or rape tries to prove innocence by showing that he is not the father of the child born to the complainant.
3. A child is born in lawful wedlock, but the husband denies paternity.
4. It is suspected that 2 newborn infants have been interchanged in a hospital.
5. A woman may simulate pregnancy and childbirth and may claim that a certain child is hers, to compel a man to marry her or to obtain dower in a dead husband's estate.
6. An individual who wishes to migrate to the United States asserts that his parents are American citizens living in the States, but it is suspected that the claim is fraudulent.

Blood tests are used only to exclude, not to prove, paternity. Examinations should be restricted to such tests that have been proved reliable and in which an adequate number of studies on heredity have been carried out. The Committee on Medicolegal Problems of the American Medical Association has recommended that the routine tests for court cases be limited to the **A** and **B** factors of the A-B-O system, the **M** and **N** factors of the M-N-S-U system, and the $\mathbf{Rh_o}$, **rh′**, **rh″**, **hr′**, and **hr″** factors of the Rh-Hr system. In private cases or under special circumstances, qualified medicolegal experts may carry out more complete examinations.

With respect to the blood groups, certain fundamental facts are apparent:

1. Blood factors cannot appear in the blood of a child unless present in the blood of one or both parents. This is true in all the blood group systems. If both parents have a definite blood structure, it is usually but not always present in all the children.
2. A person belonging to group AB cannot be the parent of a group O child.
3. A person belonging to group O cannot be the parent of a group AB child.
4. If a child is type M, the **M** factor must have been present in the blood of both the mother and father; similarly, if the child is group N, the **N** factor must have been present in the blood of both mother and father.
5. A person of type MN has derived the **M** factor from one parent and the **N** factor from the other.
6. The **M** factor cannot appear in a child unless it is present in a parent; similarly, the

N factor must be present in one or both parents in order to appear in a child.

7. If either or both parents are type M, the offspring cannot be type N. If either or both parents are type N, the offspring cannot be type M.

8. If both parents are Rh negative, there can be no Rh factor in the agglutinogens of any of the children.

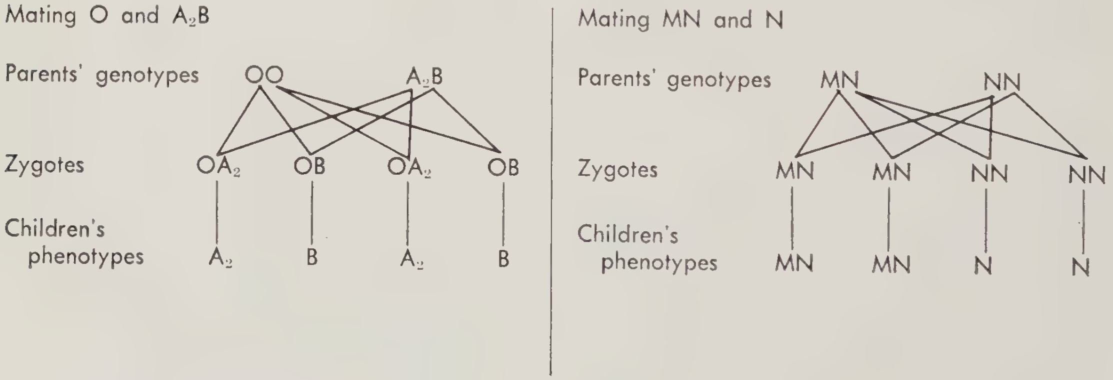
None of the Rh factors can appear in the blood of a child unless present in the blood of at least one of the parents. Since the factors of any Rh agglutinogen are inherited as a block, as Wiener has pointed out, a knowledge of the genotype possibilities in any given case is essential. A complete list of the possible matings in the Rh-Hr system and phenotypes of children is found in Table 349.

The following case has been described by Gradwohl[348] to illustrate the manner of using blood group systems A-B-O and M-N in cases of disputed paternity.

> This was a case in which the husband feared that he was not the father of his 4 yr. old child, although he stated that he had no reason to doubt the faithfulness of his wife. The father wanted this test carried out because one of his friends, evidently a trouble maker, had suggested to him in a bantering manner that he might not be the father of his child. This caused him such mental unrest that he determined to have the tests made to ease his mind. Fortunately, we were able to accomplish this purpose.
>
> The mother, child, and husband were tested in the usual manner for blood groups A-B and types M-N. The results of the tests were as follows:
>
> Mother was OMN
> Child was A_2N
> Father was A_2BN
>
> With this rather unusual combination of blood groups it was very easy to state that this man could have been the father of the child, although, as stated above, actual paternity cannot be definitely proved by these tests. Since the child possessed the agglutinogen N, which was present also in the mother's blood, the father must necessarily have had agglutinogen N in his erythrocytes. With a group O mother and a group A_2B father, the children will be either A_2 or B but not O or A_2B. (Tables 347 and 348.)
>
> Genetics for this case would be expressed as shown in Fig. 516.

With the rapid advances and discoveries in the field of blood grouping, we may look forward to a time, predicted by Landsteiner, when blood groups will be as specifically identifying as fingerprints, but that time has not yet arrived. Unger states that in approximately 50% of cases it may be impossible to establish the innocence of a man falsely accused of paternity. For example, if both the mother and the infant are group O MN and the accused man is group O MN, and all Rh phenotypes and genotypes are compatible, resort to the other factors must be made. No conclusions can be drawn on the basis of the A-B-O, M-N, and Rh-Hr tests in such a case except that the accused cannot be excluded as a possible father of the child. If, however, the accused is group AB, he is at once exonerated in this case, since no AB individual can be the parent of a group O child.

One fact must be emphasized, as Wiener has repeatedly shown, and that is that the factors of each Rh-Hr blood group agglutinogen are inherited as a block; for example, if one of the parents is type Rh_1Rh_1 and the other parent is type rh, the children possible must be ascertained by figuring the genotype of the first parent as R^1R^1, or R^1r' (Table



Children possible: A_2, B, MN, N

Children not possible: O, A_1, A_1B, A_2B, M

Fig. 516. Mating OMN × A_2BN.

314) and the genotype of the second parent as rr. The children possible to this union could be only R^1r or $r'r$; i.e., phenotype Rh$_1$rh or rh′rh but not any other phenotype. But if the Rh$_1$ parent is phenotype Rh$_1$rh, the genotypes could be R^1r, R^1R^0, or R^0r', in which case the children possible (with the other parent type rh) would be R^1r, R^0r, $r'r$, or rr. The phenotypes of the children in this mating could be Rh$_1$rh, Rh$_0$rh, rh′rh, or rh. An example of a mating of an Rh$_1$rh individual with an Rh$_2$rh person is given in Fig. 515.

In making tests to determine paternity, in which the Rh-Hr system of blood groups is used, it is vitally important that the phenotype of the parents be established. In such cases tests of the cells are made using at least all the standard anti-Rh and anti-Hr testing sera: anti-**Rh**$_0$, anti-**rh′**, anti-**rh″**, anti-**hr′**, and anti-**hr″**. It is also suggested that anti-**rh**^{w} testing serum be used as an additional aid in determining the phenotypes and possible genotypes of the parents.

Wiener[349] cited an unusual case which had been reported in detail by Franceschetti et al.[350]

> The blood specimens were submitted to Wiener and others for examination because the results of the A-B-O and M-N tests were inconclusive. The father of a pair of 5 yr. old twins, who did not look alike, while at a party noticed a boy who looked like one of his twins. Inquiry revealed that this boy had been born in the same hospital as the twins, and on the same day, and the possibility presented itself that there might have been an interchange.
>
> The boy in question and the twin he resembled both belonged to type A$_1$ MN Rh$_1$Rh$_2$ whereas the dissimilar "twin" belonged to type rh. The mother of the twins was Rh$_1$Rh$_1$. Since the genotypes of the mother were either R^1R^1 or R^1r', she could not have had an Rh-negative baby, since one of its r genes must be derived from the mother and the other from the father. Wiener states that he has been informed that the 2 boys who had been interchanged in the hospital have now been restored to their rightful parents by order of the court.
>
> Another case cited by Wiener in the same article was of a problem presented by 2 men, one married and the father of 2 children. His wife was pregnant for the third time. The problem was whether the husband or the other man was the father of the baby soon to be born. The bloods of the 2 men and the expectant mother were typed, and as soon as the baby was born her blood was tested. The results are given in Table 344.
>
> No decision in this case was possible on the basis of the A-B-O types, since the husband could be of the genotype A_2O and the other man could be BO. Nor could any decision be made on the basis of the M-N or P types. With regard to the Rh-Hr tests, the mother and baby were both type rh, genotype rr. One of the baby's r genes is obviously derived from the mother, the other from the father. If the husband were really the father, one would have to assume that he belonged to the rare genotype R^zr. On the other hand, the other man belonged to type Rh$_0$, and most Caucasoids of this type belong to genotype R^0r. The plausible conclusion was, therefore, that the other man and not the husband was the father of the baby. To solve the problem more definitely, the older 2 children were typed and both proved to be A$_2$ MN Rh$_2$rh. Accordingly, the father had to possess an R^2 gene and therefore did not belong to genotype R^zr. This definitely excluded him as the father of the third child.
>
> An interesting sidelight of the case is that the relationship between the 2 men was very amicable, and they shared all expenses equally. It was agreed that since the other man was the father of the child, he would adopt it without informing his own wife of the circumstances. In order to avoid comment from the mother's relatives, the baby was to have been sent to an institution because supposedly it was ill, and presumably the relatives were led to believe that the baby failed to recover.

Table 344

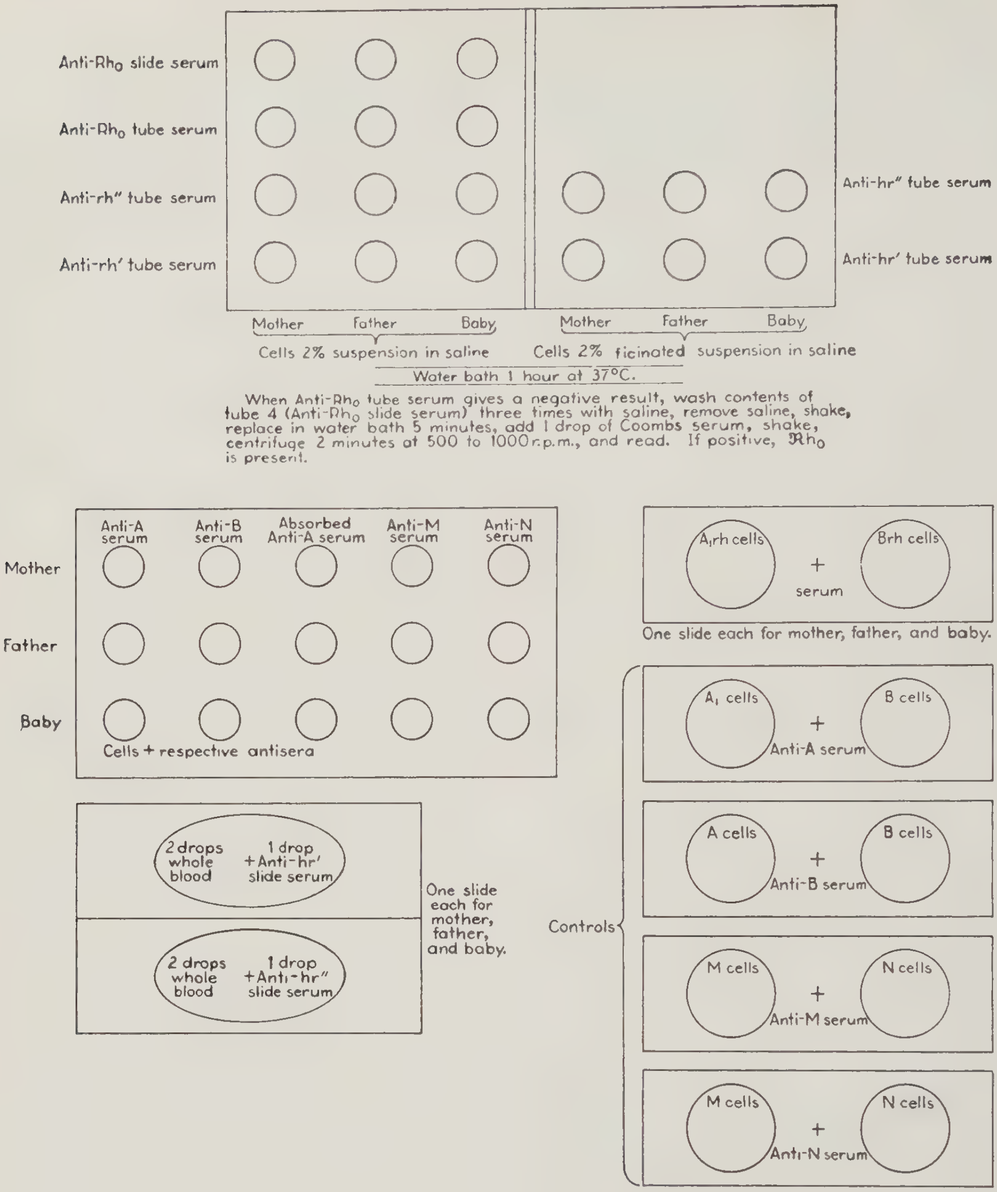
Blood of	*Group and subgroup*	*M-N type*	*Rh-Hr type*	*P factor*
Husband	A$_2$	M	Rh$_z$Rh$_0$	Negative
Wife	A$_2$	MN	rh	Negative
Baby girl	O	M	rh	Negative
Other man	B	M	Rh$_0$	Negative

Qualifications of experts

The Committee on Medicolegal Problems of the American Medical Association published 2 reports dealing with the medicolegal application of blood grouping tests[351-353] in which the importance of permitting only fully qualified experts to carry out blood tests in medicolegal cases has been stressed. This is to avoid the possibility of errors and miscarriages of justice. Serious errors have been made in the hands of unqualified laboratory personnel in performing these delicate tests.

Blood groups and disputed maternity

Blood tests may also be used in excluding disputed maternity in kidnapping cases, in cases in which newborn babies have been inadvertently interchanged in hospitals, and in immigration cases in which an individual seeking admission to the United States falsely claims certain American citizens as his parents. Wiener[354] reported 2 kidnapping cases in New York City where blood grouping tests were applied successfully.


Anti-Rh_0 slide serum
Anti-Rh_0 tube serum
Anti-rh″ tube serum
Anti-rh′ tube serum
Anti-hr″ tube serum
Anti-hr′ tube serum
Mother
Father
Baby
Cells 2% suspension in saline
Mother
Father
Baby
Cells 2% ficinated suspension in saline
Water bath 1 hour at 37°C.
Anti-A serum
Anti-B serum
Absorbed Anti-A serum
Anti-M serum
Anti-N serum
Mother
Father
Baby
Cells + respective antisera
A_1rh cells
Brh cells
+
serum
One slide each for mother, father, and baby.
2 drops whole blood
1 drop +Anti-hr′ slide serum
2 drops whole blood
1 drop +Anti-hr″ slide serum
One slide each for mother, father, and baby.
Controls
A_1 cells
B cells
+
Anti-A serum
A cells
B cells
+
Anti-B serum
M cells
N cells
+
Anti-M serum
M cells
N cells
+
Anti-N serum

When Anti-Rh_0 tube serum gives a negative result, wash contents of tube 4 (Anti-Rh_0 slide serum) three times with saline, remove saline, shake, replace in water bath 5 minutes, add 1 drop of Coombs serum, shake, centrifuge 2 minutes at 500 to 1000 r.p.m., and read. If positive, $\mathfrak{R}h_0$ is present.

Fig. 517. Setup for blood grouping tests in cases of disputed paternity.

TECHNIC OF BLOOD GROUPING TESTS FOR THE EXCLUSION OF PATERNITY*

All tests should be made at least in duplicate, using 2 sets of antisera from 2 different manufacturing sources. If these are not available, use 2 sets of antisera from the same manufacturer but from batches with different lot numbers. All tests should be read by 2 different persons without knowledge of the results obtained by one another, or the "blind" test of Wiener should be used. Refer to Fig. 517.

It is necessary to have blood specimens from (1) the mother, (2) the baby, and (3) the putative father.

Use clotted blood to obtain serum. Loosen the clot, centrifuge, and remove the supernatant serum, placing it in a properly labeled tube.

Obtain liquid whole blood by taking the specimens in dry anticoagulant (pp. 1461 and 1462) and invert 10-12 times to prevent clotting. The supernatant plasma may be used in place of serum.

If only clotted blood is submitted, make the cell suspensions (both in their own serum and in saline) by dipping a wooden applicator stick in the clot, stirring, and transferring the cells clinging to the applicator stick to the serum or the saline. In the case of serum-suspended cells, the concentration of the cells should be 50%. Centrifuge the suspension and remove enough serum to leave half cells and half serum. With saline-suspended cells the final concentration after washing should be a 2-3% or in some special tests, 5%. If the blood has been taken in an anticoagulant, transfer sufficient whole blood to saline to make the proper suspension.

---

*The author wishes to acknowledge the kindness of Dr. L. J. Unger in demonstrating these tests to me in his laboratory.

Wash the saline-suspended cells once with saline and resuspend to a 2-4% concentration.

The following antisera will be needed: standard anti-**A**, anti-**B**, anti-**A**$_1$ (absorbed group B serum) for A-B-O tests and subgroups; anti-**M**, anti-**N**, for the M-N system; and anti-**rh**′, and anti-**rh**″, and anti-**Rh**$_0$ saline agglutinating serum, and anti-**Rh**$_0$ conglutinating or slide test serum, for Rh factors; anti-**hr**′ and anti-**hr**″ to determine Rh phenotypes.

Prepare saline-suspended cells of the mother's, the baby's, and the putative father's bloods. Wash the cells once with saline and resuspend to a 2-4% concentration in saline. In testing for the Hr factors, use ficinated cells (p. 1463).

1. Test the cells for A-B-O groups by mixing the cells with anti-A and anti-**B** sera of high titer. At the same time make controls on the antisera. Carry out the technic given on pp. 1467ff.
2. Check the results in step 1 by testing the serum or plasma of the mother, baby, and putative father against known A and B cells suspended in saline (p. 1469). If results in step 2 are different from results in step 1, repeat the tests, using different antisera in step 1. When isohemolysis occurs, the results cannot be determined readily; inactivate the serum, cool to room temperature, and repeat the test.
3. Subgroup all A and AB bloods using cell suspensions against absorbed anti-**A** serum or anti-**A**$_1$ Lectin on which proper controls have been made. See p. 1471.
4. Test the cells against anti-**M** and anti-**N** sera on which proper controls have been run. This will give the M-N types. See p. 1554.
5. Prepare 3 sets of tubes in racks having 4 rows of holes and use small Kahn tubes in each case (7-8 mm. inside diameter). Each set of 4 tubes must be prepared for tests of (1) the blood of the mother, (2) the baby, and (3) the putative father. See Table 345. Saline-suspended cells washed once and resuspended in saline are used, except that ficinated cells are best for Hr testing.

Test for **rh**^{w} only when the unknown cells are shown to have **rh**′, as in types Rh_1, Rh_z, rh′, and rh_y. Do not add the anti-**rh**^{w} serum, which is in short supply, until tests for **rh**′ have been proved positive. If these tests are negative, it is not necessary to make the test for **rh**^{w}. Use 1 drop of unknown cells + 1 drop of anti-**rh**^{w} serum.

6. While these racks are standing at their respective temperatures (see specific procedures for each factor), prepare a set of tubes for Hr testing:
   (a) Prepare the cells of the mother, baby, and putative father as follows: Place 2 drops of saline-suspended cells to be tested (after washing twice in saline and resuspending to a 3-5% concentration) in each of 2 small Kahn tubes labeled, respectively, hr′ and hr″ and identified as containing the mother's blood, that of the baby, or that of the putative father.
   (b) Add to each tube 1 drop of 1% ficin diluted 1:5 with saline (p. 1463).
   (c) Shake well.
   (d) Place in a water bath at 37° C. for 15 min. for completion of the enzyme treatment of the cells.
   (e) Wash once with saline and remove the supernatant fluid.
   (f) Add 1 drop of anti-**hr**′ serum to the tubes labeled hr′ and 1 drop of anti-**hr**″ serum to the tubes labeled hr″.
   (g) Do not add the Hr antisera until tests for **rh**′ and **rh**″ have been shown to be positive. If the test for **rh**′ is positive, proceed with the test for **hr**′. If the test for **rh**″ is positive, proceed with the test for **hr**″. (It is generally thought that it is not necessary to test for **hr**′ if tests for **rh**′ are negative or to test for **hr**″ when tests for **rh**″ are negative, and that Rh-negative bloods with rare exception are **hr**′ and **hr**″ positive, but all $\overline{Rh}_0$ and $\overline{\overline{Rh}}_0$ bloods would be missed by following this procedure.)
   (h) Shake well and place in a water bath at 37° C. for 40 min.-1 hr.
7. Read the results of the Rh tests in the usual manner, as directed on p. 1517. If tests for **Rh**$_0$ are negative in tube 3, wash the cells in **tube 4** three times with saline; then, after removing all the saline proceed with the antiglobulin (Coombs) test. Refer to p. 1517. This is the test for $\mathfrak{Rh}_0$.
8. Gently dislodge the sediment in the test for **rh**^{w} and read the results macroscopically and under a scanning lens of a microscope. If the results are negative, as they are in many cases because of the nature of the **rh**^{w} antisera, proceed as follows:
   (a) Wash the contents of the tube showing negative results 3 times with saline; after

Table 345. *Setup for each blood to determine Rh type*

Back row for $\mathfrak{Rh}_0$	1 drop of unknown cells + 1 drop anti-**Rh**$_0$ conglutinating (slide test) serum
3rd row for Rh_0	1 drop of unknown cells + 1 drop anti-**Rh**$_0$ agglutinating (tube test) serum
2nd row for rh″	1 drop of unknown cells + 1 drop anti-**rh**″ serum
Front row for rh′	1 drop of unknown cells + 1 drop anti-**rh**′ serum

Table 346. *Results of a test for exclusion of paternity*

	Cells plus respective antisera											
	*Anti-***A** *serum*	*Anti-***B** *serum*	*Absorbed anti-***A** *serum*	*Anti-***M** *serum*	*Anti-***N** *serum*	*Anti-***rh**′ *saline agglutinating serum*	*Anti-***rh**″ *saline agglutinating serum*	*Anti-***Rh**$_0$ *saline agglutinating serum*	*Anti-***Rh**$_0$ *conglutinating serum*	*Anti-***hr**′ *serum*	*Anti-***hr**″ *serum*	*Blood group (phenotype)*
Mother	+	–	–	+	–	–	–	–	$\frac{-}{-}$	+*	+*	A$_2$ M rh
Father	–	+		–	+	+	+	+	Not necessary	+	+	B N Rh$_z$Rh$_0$ (see Table 314)
Baby	+	–	–	+	+	–	+	+	Not necessary	+	+	A$_2$ MN Rh$_2$rh (see Table 314)

Confirmation of A-B-O grouping

	Serum + A$_1$ cells	*Serum + B cells*
Mother	–	+
Father	+	–
Baby	–	+

The following combinations of genotypes are possible in this case:
Mother: A_2A_2 *or* A_2O, MM, rr
Father: BB or BO (if this man is the father of this child, he must be BO), NN, R^zR^0 or R^1R^z, or R^zr, R^1r'', R^zr', R^0r^y
Baby: A_2A_2 or A_2O (if this man is the father of the child), MN, R^2R^0 or R^2r or R^0r''.

---

*Except for the rare types containing $\overline{\mathbf{Rh}}_0$ or $\overline{\overline{\mathbf{Rh}}}_0$, Rh-negative bloods are invariably **hr**′ and **hr**″ positive.

the last washing and centrifugation turn the tubes upside down and strike hard against a gauze pad to remove all trace of saline, but do not remove the cells.

(b) Add 1 drop of antiglobulin (Coombs) serum to each tube and shake.

(c) Centrifuge for 1 min. at 1000 r.p.m. without further incubation.

(d) Read the results of the tests after centrifugation both macroscopically over a lighted viewing box and under a scanning lens of a microscope. Report whether the results are positive or negative and the degree of agglutination if positive.

(e) In reporting results of tests for Rh-Hr factors, always report in terms of phenotypes, not genotypes, by referring to Table 314.

9. As a confirmation of the results of Hr testing, make slide tests on whole blood using anti-**hr**′ and anti-**hr**″ conglutinating (slide test) serum. These tests may be omitted if desired.

Table 346 shows results in a test made of mother, father, and baby and conclusions drawn with respect to A-B-O, M-N, and Rh-Hr types. See also Tables 347-349.

The discovery of the blood factor **hr**^{S} by Maurice Shapiro will aid in solving some of the baffling cases in the study of blood group genetics. Many commercial anti-**hr**″ sera contain unknown quantities of anti-**hr**^{S}.

For further information concerning the use of blood grouping tests in disputed paternity, refer to Wiener and Wexler,[346] Wiener,[372, 374, 374h] and Schatkin.[374a]

A summary of the various matings and possible children is shown in Tables 347-349.

SUMMARY AND CONCLUSIONS OF THE REPORT OF THE COMMITTEE ON MEDICOLEGAL PROBLEMS OF THE AMERICAN MEDICAL ASSOCIATION[355]

"Because the use of two systems of nomenclature for the Rh-Hr system leads to confusion and misunderstanding, the Committee on Medicolegal Problems of the American Medical Association recommends the adoption of a single uniform system for medicolegal reports. It is recommended that, unless and until some other convention can be agreed upon, the original Rh-Hr notations be kept as the standard and sole nomenclature for preparing approved medicolegal reports on Rh types.

"It is recommended that routine medicolegal applications of blood grouping be restricted at present to tests for the four blood groups, O, A, B, and AB of the A-B-O system; the three types M, N, and MN of the M-N-S system; and factors **Rh**$_{0}$, **rh′**, **rh″**, and **hr′** of the Rh system. Tests for additional factors and systems are considered insufficiently established for routine use at present, although they may provide evidence in particular selected cases. Such evidence should be submitted in a separate letter not part of the standard report and should be presented with proper caution. Similarly, the value of rare combinations of groups providing indication of paternity, in contrast to the conventional use of the tests to establish only nonpaternity, should be recognized in unusual cases but should be treated with caution in a separate communication not part of the definitive report."*

It must be stressed that only those especially trained in making medicolegal blood grouping tests should be permitted to make such examinations and that ability to group and match blood for transfusions does not qualify one for medicolegal work. Since the Committee on Medicolegal Problems of the American Medical Association has recommended the use of Rh-Hr nomenclature and not the C-D-E notations, one of the qualifications of the expert necessarily is that such a person must be thoroughly versed in the recommended nomenclature.

IDENTIFICATION OF BLOOD STAINS

Identification of blood stains is made in cases of alleged hit-and-run driving, rape,

Table 347. *Summary of hereditary factors M and N*

Parents	*Possible combinations of genotypes*	*Children possible*	*Children not possible*
M and M	*MM* and *MM*	M	MN, N
M and N	*MM* and *NN*	MN	N, M
M and MN	*MM* and *MN*	M, MN	N
N and N	*NN* and *NN*	N	M, MN
N and MN	*NN* and *MN*	N, MN	M
MN and MN	*MN* and *MN*	M, N, MN	—

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*From Report of the Committee on Medicolegal Problems of the American Medical Association, J.A.M.A. **164**:2036, 1957.

Text continued on p. 1612.

Table 348. *Heredity factors in A-B-O system*

Parents	*Possible combinations of genotypes*	*Children possible*	*Children not possible*
O and O	OO and OO	O	A_1, A_2, B, A_1B, A_2B
O and A_1	OO and A_1A_1 OO and A_1A_2 OO and A_1O	O, A_1, A_2	B, A_1B, A_2B
O and A_2	OO and A_2A_2 OO and A_2O	O, A_2	A_1, B, A_1B, A_2B
O and B	OO and BB OO and BO	O, B	A_1, A_2, A_1B, A_2B
O and A_1B	OO and A_1B	A_1, B	O, A_2, A_1B, A_2B
O and A_2B	OO and A_2B	A_2, B	O, A_1, A_1B, A_2B
A_1 and A_1	A_1A_1 and A_1A_1 A_1A_1 and A_1A_2 A_1A_1 and A_1O A_1A_2 and A_1A_2 A_1A_2 and A_1O A_1O and A_1O	O, A_1, A_2	B, A_1B, A_2B
A_1 and A_2	A_1A_1 and A_2A_2 A_1A_1 and A_2O A_1A_2 and A_2A_2 A_1A_2 and A_2O A_1O and A_2A_2 A_1O and A_2O	O, A_1, A_2	B, A_1B, A_2B
A_1 and B	A_1A_1 and BB A_1A_1 and BO A_1A_2 and BB A_1A_2 and BO A_1O and BB A_1O and BO	O, A_1, A_2, B, A_1B, A_2B	—
A_1 and A_1B	A_1A_1 and A_1B A_1A_2 and A_1B A_1O and A_1B	A_1, A_1B, A_2B, B	O, A_2
A_1 and A_2B	A_1A_1 and A_2B A_1A_2 and A_2B A_1O and A_2B	A_1, A_2, B, A_1B, A_2B	O
A_2 and A_2	A_2A_2 and A_2A_2 A_2A_2 and A_2O A_2O and A_2O	A_2, O	A_1, B, A_1B, A_2B
A_2 and B	A_2A_2 and BB A_2A_2 and BO A_2O and BB A_2O and BO	O, A_2, B, A_2B	A_1, A_1B
A_2 and A_1B	A_2A_2 and A_1B A_2O and A_1B	A_1, B, A_2B	O, A_2, A_1B
A_2 and A_2B	A_2A_2 and A_2B A_2O and A_2B	A_2, B, A_2B	O, A_1, A_1B
B and B	BB and BB BB and BO BO and BO	B, O	A_1, A_2, A_1B, A_2B
B and A_1B	BB and A_1B BO and A_1B	A_1, B, A_1B	O, A_2, A_2B
B and A_2B	BB and A_2B BO and A_2B	A_2, B, A_2B	O, A_1, A_1B
A_1B and A_1B	A_1B and A_1B	A_1, B, A_1B	O, A_2, A_2B
A_1B and A_2B	A_1B and A_2B	A_1, B, A_1B, A_2B	O, A_2
A_2B and A_2B	A_2B and A_2B	A_2, B, A_2B	O, A_1, A_1B

Table 349. *Heredity of Rh-Hr types**

	Mating	*Rh-Hr types possible in children*	*Rh-Hr types impossible in children*
1.	rh × rh	rh	rh', rh", rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
2.	rh × rh'rh	rh, rh'rh	rh'rh', rh", rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
3.	rh × rh'rh'	rh'rh	rh, rh'rh', rh", rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
4.	rh × rh"rh	rh, rh"rh	rh', rh"rh", rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
5.	rh × rh"rh"	rh"rh	rh, rh', rh"rh", rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
6.	rh × rh$_y$rh	rh, rh'rh, rh"rh, rh$_y$rh	rh'rh', rh"rh", rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
7.	rh × rh$_y$rh'	rh'rh, rh$_y$rh	rh, rh'rh', rh", rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
8.	rh × rh$_y$rh"	rh"rh, rh$_y$rh	rh, rh', rh"rh", rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
9.	rh × rh$_y$rh$_y$	rh$_y$rh	rh, rh', rh", rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
10.	rh'rh × rh'rh	rh, rh'rh, rh'rh'	rh", rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
11.	rh'rh × rh'rh'	rh'rh, rh'rh'	rh, rh", rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
12.	rh'rh' × rh'rh'	rh'rh'	rh, rh'rh, rh", rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
13.	rh'rh × rh"rh	rh, rh'rh, rh"rh, rh$_y$rh	rh'rh', rh"rh", rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
14.	rh'rh' × rh"rh	rh'rh, rh$_y$rh	rh, rh'rh', rh", rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
15.	rh'rh × rh"rh"	rh"rh, rh$_y$rh	rh, rh', rh"rh", rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
16.	rh'rh' × rh"rh"	rh$_y$rh	rh, rh', rh", rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
17.	rh'rh × rh$_y$rh	rh, rh'rh, rh'rh', rh"rh, rh$_y$rh, rh$_y$rh'	rh"rh", rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
18.	rh'rh' × rh$_y$rh	rh'rh, rh'rh', rh$_y$rh, rh$_y$rh'	rh, rh", rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
19.	rh'rh × rh$_y$rh'	rh'rh, rh'rh', rh$_y$rh, rh$_y$rh'	rh, rh", rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
20.	rh'rh' × rh$_y$rh'	rh'rh', rh$_y$rh'	rh, rh'rh, rh", rh$_y$rh, rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
21.	rh'rh × rh$_y$rh"	rh"rh, rh$_y$rh, rh$_y$rh'	rh, rh', rh"rh", rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
22.	rh'rh' × rh$_y$rh"	rh$_y$rh, rh$_y$rh'	rh, rh', rh", rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
23.	rh'rh × rh$_y$rh$_y$	rh$_y$rh, rh$_y$rh'	rh, rh', rh", rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
24.	rh'rh' × rh$_y$rh$_y$	rh$_y$rh'	rh, rh', rh", rh$_y$rh, rh$_y$rh", rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
25.	rh"rh × rh"rh	rh, rh"rh, rh"rh"	rh', rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
26.	rh"rh × rh"rh"	rh"rh, rh"rh"	rh, rh', rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
27.	rh"rh" × rh"rh"	rh"rh"	rh, rh', rh"rh, rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
28.	rh"rh × rh$_y$rh	rh, rh'rh, rh"rh, rh"rh", rh$_y$rh, rh$_y$rh"	rh'rh', rh$_y$rh', rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
29.	rh"rh" × rh$_y$rh	rh"rh, rh"rh", rh$_y$rh, rh$_y$rh"	rh, rh', rh$_y$rh', rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
30.	rh"rh × rh$_y$rh'	rh'rh, rh$_y$rh, rh$_y$rh"	rh, rh'rh', rh", rh$_y$rh', rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
31.	rh"rh" × rh$_y$rh'	rh$_y$rh, rh$_y$rh"	rh, rh', rh", rh$_y$rh', rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
32.	rh"rh × rh$_y$rh"	rh"rh, rh"rh", rh$_y$rh, rh$_y$rh"	rh, rh', rh$_y$rh', rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
33.	rh"rh" × rh$_y$rh"	rh"rh", rh$_y$rh"	rh, rh', rh"rh, rh$_y$rh, rh$_y$rh', rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
34.	rh"rh × rh$_y$rh$_y$	rh$_y$rh, rh$_y$rh"	rh, rh', rh", rh$_y$rh', rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
35.	rh"rh" × rh$_y$rh$_y$	rh$_y$rh"	rh, rh', rh", rh$_y$rh, rh$_y$rh', rh$_y$rh$_y$, Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
36.	rh$_y$rh × rh$_y$rh	rh, rh'rh, rh'rh', rh"rh, rh"rh", rh$_y$rh, rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$	Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
37.	rh$_y$rh × rh$_y$rh'	rh'rh, rh'rh', rh$_y$rh, rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$	rh, rh", Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
38.	rh$_y$rh × rh$_y$rh"	rh"rh, rh"rh", rh$_y$rh, rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$	rh, rh', Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
39.	rh$_y$rh × rh$_y$rh$_y$	rh$_y$rh, rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$	rh, rh', rh", Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
40.	rh$_y$rh' × rh$_y$rh'	rh'rh', rh$_y$rh', rh$_y$rh$_y$	rh, rh'rh, rh", rh$_y$rh, rh$_y$rh", Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
41.	rh$_y$rh' × rh$_y$rh"	rh$_y$rh, rh$_y$rh', rh$_y$rh", rh$_y$rh$_y$	rh, rh', rh", Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
42.	rh$_y$rh' × rh$_y$rh$_y$	rh$_y$rh', rh$_y$rh$_y$	rh, rh', rh", rh$_y$rh, rh$_y$rh", Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
43.	rh$_y$rh" × rh$_y$rh"	rh"rh", rh$_y$rh", rh$_y$rh$_y$	rh, rh', rh"rh, rh$_y$rh, rh$_y$rh', Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
44.	rh$_y$rh" × rh$_y$rh$_y$	rh$_y$rh", rh$_y$rh$_y$	rh, rh', rh", rh$_y$rh, rh$_y$rh', Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
45.	rh$_y$rh$_y$ × rh$_y$rh$_y$	rh$_y$rh$_y$	rh, rh', rh", rh$_y$rh, rh$_y$rh', rh$_y$rh", Rh$_o$, Rh$_1$, Rh$_2$, Rh$_z$
46.	rh × Rh$_o$	rh, Rh$_o$	rh', rh", rh$_y$, Rh$_1$, Rh$_2$, Rh$_z$
47.	rh × Rh$_1$rh	rh, rh'rh, Rh$_o$, Rh$_1$rh	rh'rh', rh", rh$_y$, Rh$_1$Rh$_1$, Rh$_2$, Rh$_z$
48.	rh × Rh$_1$Rh$_1$	rh'rh, Rh$_1$rh	rh, rh'rh', rh", rh$_y$, Rh$_o$, Rh$_1$Rh$_1$, Rh$_2$, Rh$_z$

*From Wiener, A. S.: Bull. WHO 3:265, 1950.

Continued on next page.

Table 349. *Heredity of Rh-Hr types*—cont'd

	Mating	*Rh-Hr types possible in children*	*Rh-Hr types impossible in children*
49.	rh × Rh_2rh	rh, rh''rh, Rh_0, Rh_2rh	rh', rh''rh'', rh_y, Rh_1, Rh_2Rh_2, Rh_z
50.	rh × Rh_2Rh_2	rh''rh, Rh_2rh	rh, rh', rh''rh'', rh_y, Rh_0, Rh_1, Rh_2Rh_2, Rh_z
51.	rh × Rh_zRh_0	rh, rh'rh, rh''rh, rh_yrh, Rh_0, Rh_1rh, Rh_2rh, Rh_zRh_0	rh'rh', rh''rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_1Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
52.	rh × Rh_zRh_1	rh'rh, rh_yrh, Rh_1rh, Rh_zRh_0	rh, rh'rh', rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
53.	rh × Rh_zRh_2	rh''rh, rh_yrh, Rh_2rh, Rh_zRh_0	rh, rh', rh''rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
54.	rh × Rh_zRh_z	rh_yrh, Rh_zRh_0	rh, rh', rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
55.	rh'rh × Rh_0	rh, rh'rh, Rh_0, Rh_1rh	rh'rh', rh'', rh_y, Rh_1,Rh_1, Rh_2, Rh_z
56.	rh'rh' × Rh_0	rh'rh, Rh_1rh	rh, rh'rh', rh'', rh_y, Rh_0, Rh_1Rh_1, Rh_2, Rh_z
57.	rh'rh × Rh_1rh	rh, rh'rh, rh'rh', Rh_0, Rh_1rh, Rh_1Rh_1	rh'', rh_y, Rh_2, Rh_z
58.	rh'rh' × Rh_1rh	rh'rh, rh'rh', Rh_yrh, Rh_1Rh_1	rh, rh'', rh_y, Rh_0, Rh_2, Rh_z
59.	rh'rh × Rh_1Rh_1	rh'rh, rh'rh', Rh_1rh, Rh_1Rh_1	rh, rh'', rh_y, Rh_0, Rh_2, Rh_z
60.	rh'rh' × Rh_1Rh_1	rh'rh', Rh_1Rh_1	rh, rh'rh, rh'', rh_y, Rh_0, Rh_1rh, Rh_2, Rh_z
61.	rh'rh × Rh_2rh	rh, rh'rh, rh''rh, rh_yrh. Rh_0, Rh_1rh, Rh_2rh, Rh_zRh_0	rh'rh', rh''rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_1Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
62.	rh'rh' × Rh_2rh	rh'rh, rh_yrh, Rh_1rh, Rh_zRh_0	rh, rh'rh', rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
63.	rh'rh × Rh_2Rh_2	rh''rh, rh_yrh, Rh_2rh, Rh_zRh_0	rh, rh', rh''rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
64.	rh'rh' × Rh_2Rh_2	rh_yrh, Rh_zRh_0	rh, rh'. rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2. Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
65.	rh'rh × Rh_zRh_0	rh. rh'rh, rh'rh', rh''rh, rh_yrh, rh_yrh', Rh_0, Rh_1rh, Rh_1Rh_1, Rh_2rh, Rh_zRh_0, Rh_zRh_1	rh''rh'', rh_yrh'', rh_yrh_y, Rh_2Rh_2, Rh_zRh_2, Rh_zRh_z
66.	rh'rh' × Rh_zRh_0	rh'rh, rh'rh', rh_yrh, rh_yrh', Rh_1rh, Rh_1Rh_1, Rh_zRh_0, Rh_zRh_1	rh, rh'', rh_yrh'', rh_yrh_y, Rh_0, Rh_2, Rh_zRh_2, Rh_zRh_z
67.	rh'rh × Rh_zRh_1	rh'rh, rh'rh', rh_yrh, rh_yrh', Rh_1rh, Rh_1Rh_1, Rh_zRh_0, Rh_zRh_1	rh, rh'', rh_yrh'', rh_yrh_y, Rh_0, Rh_2, Rh_zRh_2, Rh_zRh_z
68.	rh'rh' × Rh_zRh_1	rh'rh', rh_yrh', Rh_1Rh_1, Rh_zRh_1	rh, rh'rh, rh'', rh_yrh, rh_yrh'', rh_yrh_y, Rh_0, Rh_1rh, Rh_2, Rh_zRh_0, Rh_zRh_2, Rh_zRh_z
69.	rh'rh × Rh_zRh_2	rh''rh, rh_yrh, rh_yrh', Rh_2rh, Rh_zRh_0, Rh_zRh_1	rh, rh', rh''rh'', rh_yrh'', rh_yrh_y, Rh_0, Rh_1,Rh_2Rh_2, Rh_zRh_2, Rh_zRh_z
70.	rh'rh' × Rh_zRh_2	rh_yrh, rh_yrh', Rh_zRh_0, Rh_zRh_1	rh, rh', rh'', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_2, Rh_zRh_z
71.	rh'rh × Rh_zRh_z	rh_yrh, rh_yrh', Rh_zRh_0, Rh_zRh_1	rh, rh', rh'', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_2, Rh_zRh_z
72.	rh'rh' × Rh_zRh_z	rh_yrh', Rh_zRh_1	rh, rh', rh'', rh_yrh, rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_0, Rh_zRh_2, Rh_zRh_z
73.	rh''rh × Rh_0	rh, rh''rh, Rh_0, Rh_2rh	rh', rh''rh'', rh_y, Rh_1, Rh_2Rh_2, Rh_z
74.	rh''rh'' × Rh_0	rh''rh, Rh_2rh	rh, rh', rh''rh'', rh_y, Rh_0, Rh_1, Rh_2Rh_2, Rh_z
75.	rh''rh × Rh_1rh	rh, rh'rh, rh''rh, rh_yrh, Rh_0, Rh_1rh, Rh_2rh, Rh_zRh_0	rh'rh', rh''rh''. rh_yrh', rh_yrh'', rh_yrh_y, Rh_1Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
76.	rh''rh'' × Rh_1rh	rh''rh, rh_yrh, Rh_2rh, Rh_2Rh_0	rh, rh'. rh''rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
77.	rh''rh × Rh_1Rh_1	rh'rh, rh_yrh, Rh_1rh, Rh_zRh_0	rh, rh'rh', rh'', rh_yrh'. rh_yrh'', rh_yrh_y, Rh_0, Rh_1Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
78.	rh''rh'' × Rh_1Rh_1	rh_yrh, Rh_zRh_0	rh. rh', rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
79.	rh''rh × Rh_2rh	rh, rh''rh, rh''rh'', Rh_0, Rh_2rh, Rh_2Rh_2	rh', rh_y, Rh_1, Rh_z
80.	rh''rh'' × Rh_2rh	rh''rh, rh''rh'', Rh_2rh, Rh_2Rh_2	rh, rh', rh_y, Rh_0, Rh_1, Rh_z
81.	rh''rh × Rh_2Rh_2	rh''rh, rh''rh'', Rh_2rh, Rh_2Rh_2	rh, rh', rh_y, Rh_0, Rh_1, Rh_z
82.	rh''rh'' × Rh_2Rh_2	rh''rh'', Rh_2Rh_2	rh, rh', rh''rh, rh_y, Rh_0, Rh_1, Rh_2rh, Rh_z
83.	rh''rh × Rh_zRh_0	rh, rh'rh, rh''rh, rh''rh'', rh_yrh, rh_yrh'', Rh_0, Rh_1rh, Rh_2rh, Rh_2Rh_2, Rh_zRh_0, Rh_zRh_2	rh'rh', rh_yrh', rh_yrh_y, Rh_1Rh_1, Rh_zRh_1, Rh_zRh_z

Table 349. *Heredity of Rh-Hr types*—cont'd

	Mating	*Rh-Hr types possible in children*	*Rh-Hr types impossible in children*
84.	$rh''rh'' \times Rh_zRh_o$	$rh''rh$, $rh''rh''$, rh_yrh, rh_yrh'', Rh_2rh, Rh_2Rh_2, Rh_zRh_o, Rh_zRh_2	rh, rh', rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_zRh_1, Rh_zRh_z
85.	$rh''rh \times Rh_zRh_1$	$rh'rh$, rh_yrh, rh_yrh'', Rh_1rh, Rh_zRh_o, Rh_zRh_2	rh, $rh'rh'$, rh'', rh_yrh', rh_yrh_y, Rh_o, Rh_1Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_z
86.	$rh''rh'' \times Rh_zRh_1$	rh_yrh, rh_yrh'', Rh_zRh_o, Rh_zRh_2	rh, rh', rh'', rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_z
87.	$rh''rh \times Rh_zRh_2$	$rh''rh$, $rh''rh''$, rh_yrh, rh_yrh'', Rh_2rh, Rh_2Rh_2, Rh_zRh_o, Rh_zRh_2	rh, rh', rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_zRh_1, Rh_zRh_z
88.	$rh''rh'' \times Rh_zRh_2$	$rh''rh''$, rh_yrh'', Rh_2Rh_2, Rh_zRh_2	rh, rh', $rh''rh$, rh_yrh, rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_2rh, Rh_zRh_o, Rh_zRh_1, Rh_zRh_z
89.	$rh''rh \times Rh_zRh_z$	rh_yrh, rh_yrh'', Rh_zRh_o, Rh_zRh_2	rh, rh', rh'', rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_z
90.	$rh''rh'' \times Rh_zRh_z$	rh_yrh'', Rh_zRh_2	rh, rh', rh'', rh_yrh, rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_1, Rh_zRh_z
91.	$rh_yrh \times Rh_o$	rh, $rh'rh$, $rh''rh$, rh_yrh, Rh_o, Rh_1rh, Rh_2rh, Rh_zRh_o	$rh'rh'$, $rh''rh''$, rh_yrh', rh_yrh'', rh_yrh_y, Rh_1Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
92.	$rh_yrh' \times Rh_o$	$rh'rh$, rh_yrh, Rh_1rh, Rh_zRh_o	rh, $rh'rh'$, rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_o, Rh_1Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
93.	$rh_yrh'' \times Rh_o$	$rh''rh$, rh_yrh, Rh_2rh, Rh_zRh_o	rh, rh', $rh''rh''$, rh_yrh', rh_yrh'', rh_yrh_y, Rh_o, Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
94.	$rh_yrh_y \times Rh_o$	rh_yrh, Rh_zRh_o	rh, rh', rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
95.	$rh_yrh \times Rh_1rh$	rh, $rh'rh$, $rh'rh'$, $rh''rh$, rh_yrh, rh_yrh', Rh_o, Rh_1rh, Rh_1Rh_1, Rh_2rh, Rh_zRh_o, Rh_zRh_1	$rh''rh''$, rh_yrh'', rh_yrh_y, Rh_2Rh_2, Rh_zRh_2, Rh_zRh_z
96.	$rh_yrh \times Rh_1Rh_1$	$rh'rh$, $rh'rh'$, rh_yrh, rh_yrh', Rh_1rh, Rh_1Rh_1, Rh_zRh_o, Rh_zRh_1	rh, rh'', rh_yrh'', rh_yrh_y, Rh_o, Rh_2, Rh_zRh_2, Rh_zRh_z
97.	$rh_yrh' \times Rh_1rh$	$rh'rh$, $rh'rh'$, rh_yrh, rh_yrh', Rh_1rh, Rh_1Rh_1, Rh_zRh_o, Rh_zRh_1	rh, rh'', rh_yrh'', rh_yrh_y, Rh_o, Rh_2, Rh_zRh_2, Rh_zRh_z
98.	$rh_yrh' \times Rh_1Rh_1$	$rh'rh'$, rh_yrh', Rh_1Rh_1, Rh_zRh_1	rh, $rh'rh$, rh'', rh_yrh, rh_yrh'', rh_yrh_y, Rh_o, Rh_1rh, Rh_2, Rh_zRh_o, Rh_zRh_2, Rh_zRh_z
99.	$rh_yrh'' \times Rh_1rh$	$rh''rh$, rh_yrh, rh_yrh', Rh_2rh, Rh_zRh_o, Rh_zRh_1	rh, rh', $rh''rh''$, rh_yrh'', rh_yrh_y, Rh_o, Rh_1, Rh_2Rh_2, Rh_zRh_2, Rh_zRh_z
100.	$rh_yrh'' \times Rh_1Rh_1$	rh_yrh, rh_yrh', Rh_zRh_o, Rh_zRh_1	rh, rh', rh'', rh_yrh'', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_2, Rh_zRh_z
101.	$rh_yrh_y \times Rh_1rh$	rh_yrh, rh_yrh', Rh_zRh_o, Rh_zRh_1	rh, rh', rh'', rh_yrh'', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_2, Rh_zRh_z
102.	$rh_yrh_y \times Rh_1Rh_1$	rh_yrh', Rh_zRh_1	rh, rh', rh'', rh_yrh, rh_yrh'', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_2, Rh_zRh_z
103.	$rh_yrh \times Rh_2rh$	rh, $rh'rh$, $rh''rh$, $rh''rh''$, rh_yrh, rh_yrh'', Rh_o, Rh_1rh, Rh_2rh, Rh_2Rh_2, Rh_zRh_o, Rh_zRh_2	$rh'rh'$, rh_yrh', rh_yrh_y, Rh_1Rh_1, Rh_zRh_1, Rh_zRh_z
104.	$rh_yrh \times Rh_2Rh_2$	$rh''rh$, $rh''rh''$, rh_yrh, rh_yrh'', Rh_2rh, Rh_2Rh_2, Rh_zRh_o, Rh_zRh_2	rh, rh', rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_zRh_1, Rh_zRh_z
105.	$rh_yrh' \times Rh_2rh$	$rh'rh$, rh_yrh, rh_yrh'', Rh_1rh, Rh_zRh_o, Rh_zRh_2	rh, $rh'rh'$, rh'', rh_yrh', rh_yrh_y, Rh_o, Rh_1Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_z
106.	$rh_yrh' \times Rh_2Rh_2$	rh_yrh, rh_yrh'', Rh_zRh_o, Rh_zRh_2	rh, rh', rh'', rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_z
107.	$rh_yrh'' \times Rh_2rh$	$rh''rh$, $rh''rh''$, rh_yrh, rh_yrh'', Rh_2rh, Rh_2Rh_2, Rh_zRh_o, Rh_zRh_2	rh, rh', rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_zRh_1, Rh_zRh_z
108.	$rh_yrh'' \times Rh_2Rh_2$	$rh''rh''$, rh_yrh'', Rh_2Rh_2, Rh_zRh_2	rh, rh', $rh''rh$, rh_yrh, rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_2rh, Rh_zRh_o, Rh_zRh_1, Rh_zRh_z
109.	$rh_yrh_y \times Rh_2rh$	rh_yrh, rh_yrh'', Rh_zRh_o, Rh_zRh_2	rh, rh', rh'', rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_z
110.	$rh_yrh_y \times Rh_2Rh_2$	rh_yrh'', Rh_zRh_2	rh, rh', rh'', rh_yrh, rh_yrh', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_1, Rh_zRh_z
111.	$rh_yrh \times Rh_zRh_o$	rh, $rh'rh$, $rh'rh'$, $rh''rh$, $rh''rh''$, rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_o, Rh_1rh, Rh_1Rh_1, Rh_2rh, Rh_2Rh_2, Rh_zRh_o, Rh_zRh_1 Rh_zRh_2, Rh_zRh_z	None

Continued on next page.

Table 349. *Heredity of Rh-Hr types*—cont'd

	Mating	*Rh-Hr types possible in children*	*Rh-Hr types impossible in children*
112.	$rh_yrh \times Rh_zRh_1$	rh′rh, rh′rh′, rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_1rh, Rh_1Rh_1, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z	rh, rh″, Rh_o, Rh_2
113.	$rh_yrh \times Rh_zRh_2$	rh″rh, rh″rh,″ rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_2rh, Rh_2Rh_2, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z	rh, rh′, Rh_o, Rh_1
114.	$rh_yrh \times Rh_zRh_z$	rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z	rh, rh′, rh″, Rh_o, Rh_1, Rh_2
115.	$rh_yrh' \times Rh_zRh_o$	rh′rh, rh′rh′, rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_1rh, Rh_1Rh_1, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z	rh, rh″, Rh_o, Rh_2
116.	$rh_yrh' \times Rh_zRh_1$	rh′rh′, rh_yrh', rh_yrh_y, Rh_1Rh_1, Rh_zRh_1, Rh_zRh_z	rh, rh′rh, rh″, rh_yrh, rh_yrh'', Rh_o, Rh_1rh, Rh_zRh_o, Rh_zRh_2
117.	$rh_yrh' \times Rh_zRh_2$	rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z	rh, rh′, rh″, Rh_o, Rh_1, Rh_2
118.	$rh_yrh' \times Rh_zRh_z$	rh_yrh', rh_yrh_y, Rh_zRh_1, Rh_zRh_z	rh, rh′, rh″, rh_yrh, rh_yrh'', Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_2
119.	$rh_yrh'' \times Rh_zRh_o$	rh″rh, rh″rh″, rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_2rh, Rh_2Rh_2, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z	rh, rh′, Rh_o, Rh_1
120.	$rh_yrh'' \times Rh_zRh_1$	rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z	rh, rh′, rh″, Rh_o, Rh_1, Rh_2
121.	$rh_yrh'' \times Rh_zRh_2$	rh″rh″, rh_yrh'', rh_yrh_y, Rh_2Rh_2, Rh_zRh_2, Rh_zRh_z	rh, rh′, rh″rh, rh_yrh, rh_yrh', Rh_o, Rh_1, Rh_2rh, Rh_zRh_o, Rh_zRh_1
122.	$rh_yrh'' \times Rh_zRh_z$	rh_yrh'', rh_yrh_y, Rh_zRh_2, Rh_zRh_z	rh, rh′, rh″, rh_yrh, rh_yrh', Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_1
123.	$rh_yrh_y \times Rh_zRh_o$	rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z	rh, rh′, rh″, Rh_o, Rh_1, Rh_2
124.	$rh_yrh_y \times Rh_zRh_1$	rh_yrh', rh_yrh_y, Rh_zRh_1, Rh_zRh_z	rh, rh′, rh″, rh_yrh, rh_yrh'', Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_2
125.	$rh_yrh_y \times Rh_zRh_2$	rh_yrh'', rh_yrh_y, Rh_zRh_2, Rh_zRh_z	rh, rh′, rh″, rh_yrh, rh_yrh', Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_1
126.	$rh_yrh_y \times Rh_zRh_z$	rh_yrh_y, Rh_zRh_z	rh, rh′, rh″, rh_yrh, rh_yrh', rh_yrh'', Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2
127.	$Rh_o \times Rh_o$	rh, Rh_o	rh′, rh″, rh_y, Rh_1, Rh_2, Rh_z
128.	$Rh_o \times Rh_1rh$	rh, rh′rh, Rh_o, Rh_1rh	rh′rh′, rh″, rh_y, Rh_1Rh_1, Rh_2, Rh_z
129.	$Rh_o \times Rh_1Rh_1$	rh′rh, Rh_1rh	rh, rh′rh′, rh″, rh_y, Rh_o, Rh_1Rh_1, Rh_2, Rh_z
130.	$Rh_o \times Rh_2rh$	rh, rh″rh, Rh_o, Rh_2rh	rh′, rh″rh″, rh_y, Rh_1, Rh_2Rh_2, Rh_z
131.	$Rh_o \times Rh_2Rh_2$	rh″rh, Rh_2rh	rh, rh′, rh″rh″, rh_y, Rh_o, Rh_1, Rh_2Rh_2, Rh_z
132.	$Rh_o \times Rh_zRh_o$	rh, rh′rh, rh″rh, rh_yrh, Rh_o, Rh_1rh, Rh_2rh, Rh_zRh_o	rh′rh′, rh″rh″, rh_yrh', rh_yrh'', rh_yrh_y, Rh_1Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
133.	$Rh_o \times Rh_zRh_1$	rh′rh, rh_yrh, Rh_1rh, Rh_zRh_o	rh, rh′rh′, rh″, rh_yrh', rh_yrh'', rh_yrh_y, Rh_o, Rh_1Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
134.	$Rh_o \times Rh_zRh_2$	rh″rh, rh_yrh, Rh_2rh, Rh_zRh_o	rh, rh′, rh″rh″, rh_yrh', rh_yrh'', rh_yrh_y, Rh_o, Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
135.	$Rh_o \times Rh_zRh_z$	rh_yrh, Rh_zRh_o	rh, rh′, rh″, rh_yrh', rh_yrh'', rh_yrh_y, Rh_o, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
136.	$Rh_1rh \times Rh_1rh$	rh, rh′rh, rh′rh′, Rh_o, Rh_1rh, Rh_1Rh_1	rh″, rh_y, Rh_2, Rh_z
137.	$Rh_1rh \times Rh_1Rh_1$	rh′rh, rh′rh′, Rh_1rh, Rh_1Rh_1	rh, rh″, rh_y, Rh_o, Rh_2, Rh_z
138.	$Rh_1Rh_1 \times Rh_1Rh_1$	rh′rh′, Rh_1Rh_1	rh, rh′rh, rh″, rh_y, Rh_o, Rh_1rh, Rh_2, Rh_z
139.	$Rh_1rh \times Rh_2rh$	rh, rh′rh, rh″rh, rh_yrh, Rh_o, Rh_1rh, Rh_2rh, Rh_zRh_o	rh′rh′, rh″rh″, rh_yrh', rh_yrh'', rh_yrh_y, Rh_1Rh_1 Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z
140.	$Rh_1rh \times Rh_2Rh_2$	rh″rh, rh_yrh, Rh_2rh, Rh_zRh_o	rh, rh′, rh″rh″, rh_yrh', rh_yrh'', rh_yrh_y, Rh_o, Rh_1, Rh_2Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z

Table 349. *Heredity of Rh-Hr types*—cont'd

	Mating	*Rh-Hr types possible in children*	*Rh-Hr types impossible in children*
141.	$Rh_1Rh_1 \times Rh_2rh$	$rh'rh, rh_yrh, Rh_1rh, Rh_zRh_0$	$rh, rh'rh', rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z$
142.	$Rh_1Rh_1 \times Rh_2Rh_2$	rh_yrh, Rh_zRh_0	$rh, rh', rh'', rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z$
143.	$Rh_1rh \times Rh_zRh_0$	$rh, rh'rh, rh'rh', rh''rh, rh_yrh, rh_yrh', Rh_0, Rh_1rh, Rh_1Rh_1, Rh_2rh, Rh_zRh_0, Rh_zRh_1$	$rh''rh'', rh_yrh'', rh_yrh_y, Rh_2Rh_2, Rh_zRh_2, Rh_zRh_z$
144.	$Rh_1Rh_1 \times Rh_zRh_0$	$rh'rh, rh'rh', rh_yrh, rh_yrh', Rh_1rh, Rh_1Rh_1, Rh_zRh_0, Rh_zRh_1$	$rh, rh'', rh_yrh'', rh_yrh_y, Rh_0, Rh_2, Rh_zRh_2, Rh_zRh_z$
145.	$Rh_1rh \times Rh_zRh_1$	$rh'rh, rh'rh', rh_yrh, rh_yrh', Rh_1rh, Rh_1Rh_1, Rh_zRh_0, Rh_zRh_1$	$rh, rh'', rh_yrh'', rh_yrh_y, Rh_0, Rh_2, Rh_zRh_2, Rh_zRh_z$
146.	$Rh_1Rh_1 \times Rh_zRh_1$	$rh'rh', rh_yrh', Rh_1Rh_1, Rh_zRh_1$	$rh, rh'rh, rh'', rh_yrh, rh_yrh'', rh_yrh_y, Rh_0, Rh_1rh, Rh_2, Rh_zRh_0, Rh_zRh_2, Rh_zRh_z$
147.	$Rh_1rh \times Rh_zRh_2$	$rh''rh, rh_yrh, rh_yrh', Rh_2rh, Rh_zRh_0, Rh_zRh_1$	$rh, rh', rh''rh'', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2Rh_2, Rh_zRh_2, Rh_zRh_z$
148.	$Rh_1Rh_1 \times Rh_zRh_2$	$rh_yrh, rh_yrh', Rh_zRh_0, Rh_zRh_1$	$rh, rh', rh'', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_2, Rh_zRh_z$
149.	$Rh_1rh \times Rh_zRh_z$	$rh_yrh, rh_yrh', Rh_zRh_0, Rh_zRh_1$	$rh, rh', rh'', rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_2, Rh_zRh_z$
150.	$Rh_1Rh_1 \times Rh_zRh_z$	rh_yrh', Rh_zRh_1	$rh, rh', rh'', rh_yrh, rh_yrh'', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_0, Rh_zRh_2, Rh_zRh_z$
151.	$Rh_2rh \times Rh_2rh$	$rh, rh''rh, rh''rh'', Rh_0, Rh_2rh, Rh_2Rh_2$	rh', rh_y, Rh_1, Rh_z
152.	$Rh_2rh \times Rh_2Rh_2$	$rh''rh, rh''rh'', Rh_2rh, Rh_2Rh_2$	$rh, rh', rh_y, Rh_0, Rh_1, Rh_z$
153.	$Rh_2Rh_2 \times Rh_2Rh_2$	$rh''rh'', Rh_2Rh_2$	$rh, rh', rh''rh, rh_y, Rh_0, Rh_1, Rh_2rh, Rh_z$
154.	$Rh_2rh \times Rh_zRh_0$	$rh, rh'rh, rh''rh, rh''rh'', rh_yrh, rh_yrh'', Rh_0, Rh_1rh, Rh_2rh, Rh_2Rh_2, Rh_zRh_0, Rh_zRh_2$	$rh'rh', rh_yrh', rh_yrh_y, Rh_1Rh_1, Rh_zRh_1, Rh_zRh_z$
155.	$Rh_2Rh_2 \times Rh_zRh_0$	$rh''rh, rh''rh'', rh_yrh, rh_yrh'', Rh_2rh, Rh_2Rh_2, Rh_zRh_0, Rh_zRh_2$	$rh, rh', rh_yrh', rh_yrh_y, Rh_0, Rh_1, Rh_zRh_1, Rh_zRh_z$
156.	$Rh_2rh \times Rh_zRh_1$	$rh'rh, rh_yrh, rh_yrh'', Rh_1rh, Rh_zRh_0, Rh_zRh_2$	$rh, rh'rh', rh'', rh_yrh', rh_yrh_y, Rh_0, Rh_1Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_z$
157.	$Rh_2Rh_2 \times Rh_zRh_1$	$rh_yrh, rh_yrh'', Rh_zRh_0, Rh_zRh_2$	$rh, rh', rh'', rh_yrh', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_z$
158.	$Rh_2rh \times Rh_zRh_2$	$rh''rh, rh''rh'', rh_yrh, rh_yrh'', Rh_2rh, Rh_2Rh_2, Rh_zRh_0, Rh_zRh_2$	$rh, rh', rh_yrh', rh_yrh_y, Rh_0, Rh_1, Rh_zRh_1, Rh_zRh_z$
159.	$Rh_2Rh_2 \times Rh_zRh_2$	$rh''rh'', rh_yrh'', Rh_2Rh_2, Rh_zRh_2$	$rh, rh', rh''rh, rh_yrh, rh_yrh', rh_yrh_y, Rh_0, Rh_1, Rh_2rh, Rh_zRh_0, Rh_zRh_1, Rh_zRh_z$
160.	$Rh_2rh \times Rh_zRh_z$	$rh_yrh, rh_yrh'', Rh_zRh_0, Rh_zRh_2$	$rh, rh', rh'', rh_yrh', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_1, Rh_zRh_z$
161.	$Rh_2Rh_2 \times Rh_zRh_z$	rh_yrh'', Rh_zRh_2	$rh, rh', rh'', rh_yrh, rh_yrh', rh_yrh_y, Rh_0, Rh_1, Rh_2, Rh_zRh_0, Rh_zRh_1, Rh_zRh_z$
162.	$Rh_zRh_0 \times Rh_zRh_0$	$rh, rh'rh, rh'rh', rh''rh, rh''rh'', rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_0, Rh_1rh, Rh_1Rh_1, Rh_2rh, Rh_2Rh_2, Rh_zRh_0, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z$	None
163.	$Rh_zRh_0 \times Rh_zRh_1$	$rh'rh, rh'rh', rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_1rh, Rh_1Rh_1, Rh_zRh_0, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z$	rh, rh'', Rh_0, Rh_2
164.	$Rh_zRh_0 \times Rh_zRh_2$	$rh''rh, rh''rh'', rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_2rh, Rh_2Rh_2, Rh_zRh_0, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z$	rh, rh', Rh_0, Rh_1
165.	$Rh_zRh_0 \times Rh_zRh_z$	$rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_zRh_0, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z$	$rh, rh', rh'', Rh_0, Rh_1, Rh_2$
166.	$Rh_zRh_1 \times Rh_zRh_1$	$rh'rh', rh_yrh', rh_yrh_y, Rh_1Rh_1, Rh_zRh_1, Rh_zRh_z$	$rh, rh'rh, rh'', rh_yrh, rh_yrh'', Rh_0, Rh_1rh, Rh_2, Rh_zRh_0, Rh_zRh_2$

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Table 349. *Heredity of Rh-Hr types*—cont'd

	Mating	*Rh-Hr types possible in children*	*Rh-Hr types impossible in children*
167.	$Rh_zRh_1 \times Rh_zRh_2$	rh_yrh, rh_yrh', rh_yrh'', rh_yrh_y, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2, Rh_zRh_z	rh, rh', rh'', Rh_o, Rh_1, Rh_2
168.	$Rh_zRh_1 \times Rh_zRh_z$	rh_yrh', rh_yrh_y, Rh_zRh_1, Rh_zRh_z	rh, rh', rh'', rh_yrh, rh_yrh'', Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_2
169.	$Rh_zRh_2 \times Rh_zRh_2$	$rh''rh''$, rh_yrh'', rh_yrh_y, Rh_2Rh_2, Rh_zRh_2, Rh_zRh_z	rh, rh', $rh''rh$, rh_yrh, rh_yrh', Rh_o, Rh_1, Rh_2rh, Rh_zRh_o, Rh_zRh_1
170.	$Rh_zRh_2 \times Rh_zRh_z$	rh_yrh'', rh_yrh_y, Rh_zRh_2, Rh_zRh_z	rh, rh', rh'', rh_yrh, rh_yrh', Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_1
171.	$Rh_zRh_z \times Rh_zRh_z$	rh_yrh_y, Rh_zRh_z	rh, rh', rh'', rh_yrh, rh_yrh', rh_yrh'', Rh_o, Rh_1, Rh_2, Rh_zRh_o, Rh_zRh_1, Rh_zRh_2

murder, or wherever stains suspected of being blood are found. The specimens are received in the laboratory on a variety of objects. These must all be carefully labeled in indelible ink, and the specimens must be kept for possible later presentation in court. This work must be done only by those properly qualified to perform the tests. It is much more difficult to perform these tests than is ordinary blood grouping. It requires much practice to develop the accuracy required in medicolegal examinations. At least 50 known blood stains should be correctly identified before a person is qualified to perform this work in medicolegal cases.

Gradwohl[356] pointed out the importance of maintaining the integrity of the chain of evidence. All evidence should be collected by members of the laboratory staff and by no one else, and the laboratory staff must work in close harmony with the homicide squad. Material should be brought to the director's office, routinely entered in the laboratory book of entry, with time, names, and other pertinent data all written, and receipts properly given by those who receive the specimen. Full written data must be compiled covering the name of the person who brought in the specimen, the time brought in, and the date. A receipt should be given to the individual who brings in the material, and that person should also make a written statement that he has brought in the material. One must be prepared to submit all this written evidence during the trial of the case.

Identifying a blood stain presents 3 problems: (1) Is the stain produced by blood? (2) If so, is this human blood? (3) To what blood group does it belong?

Tests to prove that stains are blood

Chemical tests and spectroscopic examination

Chemical tests and spectroscopic examination will definitely establish the identity of a stain as blood or not blood.

Benzidine test

The benzidine test may be made directly on the stained area or on an extract of or scrapings from the stain. In any case control tests must be made of the unstained area at the same time that tests are made of the stain. All glassware used in the test must be new or thoroughly cleaned and dried in a hot-air oven.

1. Prepare an extract in saline of the material suspected of being blood. If this is in the form of scrapings, use a very small amount of the scrapings without dissolving, or, if preferred, dissolve in a **minimum** quantity of physiologic saline.
2. If the stain is on cloth, cut out the stained portion, place it in a small (7 mm. inside diameter), tube, and add 1 or 2 drops of saline. Mark the area from which the stain has been removed, using indelible ink.
3. Prepare the control specimens from the unstained material in a similar manner. If the control specimens show a positive reaction, no further work may be done, as the test in such cases would be unreliable.
4. Prepare a saturated solution of benzidine, special for blood, in glacial acetic acid* by placing a small amount of benzidine in a clean small tube, free from blood, and adding about 0.5-1 ml. glacial acetic acid to it. Shake vigorously. If all the crystals dissolve, add more benzidine until the acetic acid will not take up more of the benzidine. This solution must be prepared fresh for each test.
5. Add an equal volume of 3% hydrogen peroxide and mix thoroughly. If this solution turns green or blue, discard and prepare a fresh solution, using different glassware, because the green or blue color in the reagent may have been caused by blood either in the glassware or in the chemicals used to prepare the solution.
6. Place a drop of the extract or the scrapings on a **new** clean glass slide and add a drop of the benzidine reagent. This reagent may be added with a wooden applicator stick or with a small glass rod. Or add the reagent directly to the stained area without any preliminary preparation.

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*The benzidine dihydrochloride test, p. 1835, may be used instead.

A green or blue color indicates the presence of blood. If this color fails to appear, repeat the test. If no color appears, the stain was not derived from blood and the test is terminated at this point. If the specimen from the unstained portion of the material shows a blue or green color with this test, the results of the test on the stained area are valueless and no further work may be done.

Phenolphthalein test (reduced phenolphthalein test)

Refer to p. 1834 for details of this test.

Spectroscopic examination of blood stains

Although the spectroscopic examination of blood is not routine practice, it is used at times in testing denatured blood.

The spectroscopic examination is based upon the fact that when a solution of blood is examined in a spectroscope, certain black bands, called absorption bands, will be formed in the solar spectrum. Especially constructed spectroscopes are used. These instruments are so made that the absorption properties of translucent color fluids can be observed on the solar spectrum. There are many varieties of spectroscopes—a large laboratory spectroscope, a smaller direct vision spectroscope, and a microspectroscope that can be attached to an ordinary microscope. The last instrument is used only when a minute quantity of specimen is available.

1. Dissolve the stain in distilled water. The solution must be quite dilute.
2. Place the solution in a small glass chamber with parallel sides so arranged that rays of light will pass directly through it; then place the chamber containing the dissolved stain in the spectroscope and adjust the instrument so that the spectrum is clearly visible. The blood will absorb some of the light rays from the spectrum, producing characteristic absorption bands, which vary with the type of blood pigment present.

Oxyhemoglobin: 2 bands in the yellow between the Fraunhofer lines D and E.

Hemoglobin: A broad band between D and E, with a small rim over D on the red side of the spectrum.

Carboxyhemoglobin: Similar to oxyhemoglobin. Addition of ammonium sulfide reduces oxyhemoglobin to hemoglobin but leaves carboxyhemoglobin unchanged.

Methemoglobin: A dark band in the red between C and D but nearer C. If the solution is diluted properly, it splits into bands resembling bands of acid hematin.

Acid hematin: A sharp band between C and D, nearer to C, and a broad band, not clearly defined, between D and F.

Alkaline hematin: A band between C and D. It also absorbs the violet end of the spectrum.

Hemochromogen: A sharp, dark band between D and E and a pale, broader band over E.

Hematoporphyrin in alkaline solutions: A faint line between C and D, a broader band at D and extending toward E, a band between D and E but nearly at E, and a broad band between E and F.

Old stains, when diluted, give hemoglobin and oxyhemoglobin bands. If a weak alkali is added, alkaline hematin is formed. If a weak acid is added, acid hematin is formed. Old and decomposed stains yield positive tests for hematoporphyrin. Add strong alkali to a portion of the blood solution to obtain alkaline hematoporphyrin bands.

Fresh blood shows oxyhemoglobin bands. Add a weak solution of ammonium sulfide to obtain the spectrum of hemoglobin. Add a dilute sodium hydroxide solution for hemochromogen bands. These 2 tests prove the presence of blood and also eliminate substances of vegetable and animal origin with spectra similar to that of oxyhemoglobin.

Tests to determine whether the stains are human blood

Precipitin test

The principle of the precipitin test is based upon the work of Kraus who showed that by immunizing a rabbit with injections of a culture of a bacterium one could obtain a serum which, when added to a filtered culture of that bacterium, produces a precipitate in it. Bordet[357] and Gengou[358] went further in carrying out this immunization experiment.

Uhlenhuth worked out the same principle in relation to the precipitin test and its value as a medicolegal procedure. He injected rabbits with human blood and tested the antiserum on 19 different bloods, finding a positive reaction only with human blood. Uhlenhuth[359] published his tests on blood-stained articles such as sticks, sand, cotton, trousers, hatchet, etc. He showed that the antiserum made by injecting rabbits with human blood acted only with suspected human blood and that antiserum injected with pig's blood reacted only with pig's blood, etc. In other words, a precipitation occurs in the blood when in contact with homologous blood. Perhaps the most exhaustive of all these works was done by Nuttall,[360] who examined sera of all animals of any importance on the face of the globe.

Nuttall[360] in 1904 gave a classical account of blood relationships by means of the precipitin test. His work was done primarily to show that a common property exists in the blood of certain groups of animals, a property that has persisted throughout the ages which elapsed during our possible evolution from a common ancestor. He placed Man and Apes in one order, based on this work, the Anthropoidea. Since the publication of Nuttall's book in 1904 and the present time, there have been occasional references in the literature on the specificity of the precipitin test.[361] Gradwohl[362] found that chimpanzee blood gave a ring reaction identical to that given by human blood, with antihuman precipitin serum, and that human blood gave an identical ring reaction with antichimpanzee precipitin serum. He further showed[363] the same results using blood from the gorilla and the olive baboon *(Papio anubis)*.

In a personal communication from Lloyd M. Shupe, Columbus, Ohio, it was pointed out that some antihuman precipitin sera also give positive reactions with raccoon blood. This has been confirmed in our own laboratories.

In other words, using the "ring" precipitin test, it is impossible to differentiate the bloods of these 3 primates and man. Duncan-Taylor and Gordon,[364] by special alum absorption methods, thought it was possible to produce a precipitin serum which would exclude the blood of primates when the "ring" test is made. Their method is given on p. 1616. Gordon also examined blood stain extracts against an ox serum-absorbed antihuman precipitin serum to establish that it is primate blood, but this absorbed serum is of very poor potency.

There is no simple, practicable method available at present to distinguish between the blood stains of man and the other primates (apes, monkeys including baboons, lemurs, or nachtaapje.

Two principal methods of carrying out the precipitin test are available: the ring test technic and the flocculation test. The precipitins which are formed in animals by the injection of human blood are precipitins not for blood only but also for human protein. It is for this reason that this test can be used not only for the identification of blood but also for seminal stains, bone fragments, and meat adulteration; e.g., addition of chopped horse meat to chopped beef.

It is necessary that the antiserum used in the test be made properly and that it be tested on sufficient numbers of known positive speci-

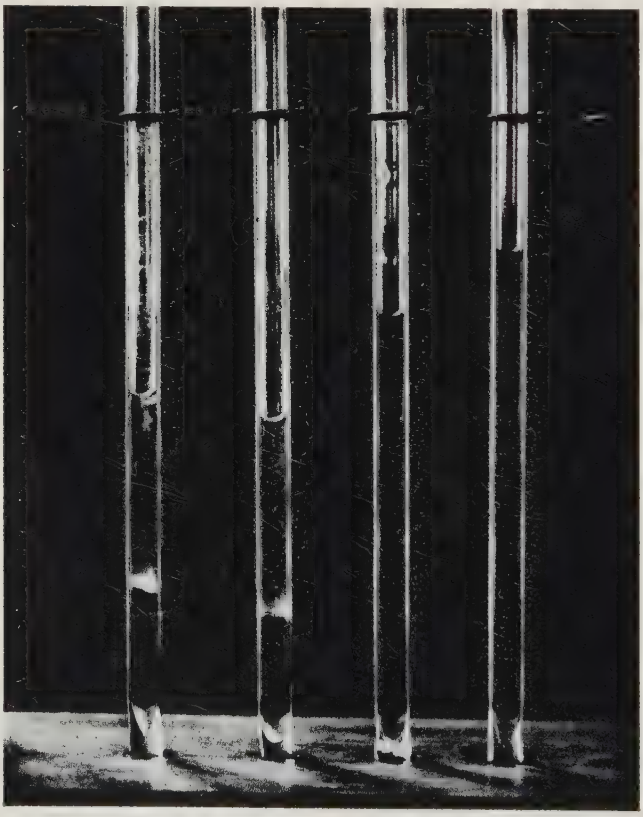

Fig. 518. Precipitin ring test. **A,** Antichimpanzee serum vs. chimpanzee blood. **B,** Antichimpanzee serum vs. human blood. **C,** Antichimpanzee serum vs. guinea pig blood. **D,** Antichimpanzee blood vs. rabbit blood. (From Gradwohl, R. B. H.: Lab. Digest 15:4, May, 1952.)

mens to warrant its use in medicolegal procedures. These antisera react with groups of mammalian bloods, and therefore in order to single out an individual of the mammalian group one must resort to high dilutions. By high dilutions is meant that the antiserum must be very potent and the material under investigation extremely well diluted for the actual test.

The use of a reliable antiserum is of course one of the most important considerations in making this test. Either prepare these antisera in the laboratory or purchase them from reliable biological houses. The latter is probably better for those who do only an occasional precipitin test. These antisera are quite stable.

In preparing the antisera it is best to employ a number of animals for the immunizations, at least 6. Animals may be immunized by injecting human blood daily over a period of 6 weeks. After this time a quantity of blood is taken from the ear and tested with a known positive human blood. If it is effective in a high dilution, the animals are bled to the point of exsanguination under aseptic precautions and the blood is collected in a sterile manner. Blood from these animals should be taken preferably several hours after the rabbits have eaten or during a period of fasting. If blood is taken from the animal immediately after a meal, the serum is likely to be opalescent.

An antiserum to be efficient should so act that 0.1 ml. when added to its respective serum-antigen in a dilution of 1:1000 produces a turbidity either at once or in 1-5 min. at the latest, with the formation of a distinct precipitate in 30-60 min.

Preparation of antihuman precipitin serum: Following is the method used by Gradwohl. It gives excellent results.

Select 6 healthy rabbits. Make 50 ml. of a 1:10 dilution of sterile human serum or plasma in saline by adding 45 ml. sterile physiologic saline to 5 ml. human serum or plasma obtained under sterile conditions.

Mark each rabbit with a tag or with indelible ink for identification, and keep complete records of each rabbit and each injection. Inject 2 ml. of 1:10 dilution of human serum or plasma into the marginal ear vein of each rabbit. Beginning on Monday and continuing through Saturday make daily injections so that a total of 6 injections is given to each rabbit.

Allow 1 wk. to elapse; then inject each rabbit with 2 ml. of 1:10 dilution of human serum or plasma **intramuscularly** (to prevent anaphylaxis), followed by daily injections for the next 5 days, giving these 5 injections intravenously as in the first week.

Allow 1 wk. rest interval. Repeat the 6 injections of 2 ml. of 1:10 dilution of human serum or plasma, giving the first injection intramuscularly and the next 5 intravenously.

Wait 1 wk. Then withdraw a small quantity of blood from each rabbit, allow to clot, loosen the clot, centrifuge, remove the supernatant serum, and test the serum to determine whether any antihuman precipitin has developed. In other words, carry out an actual precipitin test as described below to determine whether the rabbit's serum will detect the presence of human blood. Usually 1 or more of the 6 rabbits will show this antihuman precipitating antibody. Estimate by dilutions the potency of the antibody. Reject all rabbits' sera not reacting in at least a 1:1000 dilution. Carry out controls with both positive and negative specimens.

If 1 or more of these rabbits has developed an antihuman precipitin of high titer, procure the entire content of blood from the selected rabbit or rabbits by dissecting the carotid artery and bleeding into sterile 50 ml. Rockefeller centrifuge tubes, using all sterile precautions. (See p. 808 for method of bleeding rabbit from the carotid artery.)

Label the Rockefeller tubes carefully and allow the blood to clot in a slanting position. Loosen the clots by striking the tubes against the palm of the hand, refrigerate 24 hr.; then centrifuge at high speed for at least 20-30 min. to separate the serum. With all sterile precautions carefully decant the supernatant serum and place it in sterile corked tubes. Inactivate at 56° C. to destroy the natural complement and to kill air bacteria which may have entered the blood during the procuring process. Add enough Merthiolate as a preservative to give a 1:10,000 concentration (0.1 ml. of 10% solution to 100 ml. serum).

Ampule in 1 ml. quantities in sterile glass ampules, using sterile precautions. Seal in a flame, label with the number of the rabbit, the date, and titer, and store in a refrigerator.

Technic of precipitin "ring" test:

Use 5 mm. tubing with a 2 mm. bore. This is very heavy-walled narrow glass tubing. Cut it into 12 cm. lengths and seal one end in the flame. These very small caliber test tubes are used in the precipitin test. They must be clean and dry.

Soak the material to be examined in saline for some time. When stains have been exposed to high temperatures, it is necessary to soak as long as 72 hr. This extraction should be carried out in a refrigerator to avoid bacterial action and turbidity. No chemical solvents except saline should be used, regardless of how difficult it may be to obtain an extract. If the extract contains debris or is cloudy, centrifuge and use the supernatant liquid for the test or filter through a Berkefeld filter. Use a minimal quantity of saline for the extraction.

Prepare a similar extract of the **unstained portion** of the material under examination. Make the dilution about the same as that of the stained area.

Various dilutions of the extract should be used, preferably a 1:100 and 1:1000, although higher dilutions may be made and tested if desired. Dilutions of the stain of 1:1000 or higher will show only a scanty foam upon shaking.

Prepare a dilution of **known human serum** of approximately 1:100 strength by adding 1 small drop of human serum to 5 ml. saline.

Prepare a **negative control serum** by adding 1 drop of bovine or canine blood serum to 5 ml. saline (1:100).

Place antihuman precipitin serum in each of 5 small caliber tubes described above. Add this to a depth of several cm. Label the tubes (1) blood stain, (2) unstained portion, (3) known human serum, (4) bovine or canine serum, and (5) saline.

Stratify an **equal volume** of solution from the suspected stain on the serum in the first tube; use the extract from the unstained portion in the second tube, diluted human serum in the third tube, diluted canine or bovine serum in the fourth tube, and saline in the fifth tube. This is accomplished by placing the solution above the serum so that it stands about 1 mm. above it and then giving it a quick flick with the finger so that the 2 fluids come in contact with each other but do not mix.

Allow all tubes to stand at room temperature for 5 min. and read at the end of this time.

If the stain contains human blood, a white line will appear at the point of contact between the 2 liquids. This will be definite within 5 min.

This result cannot be relied upon unless the control tests are as follows: negative reaction in tube 2, positive in tube 3, and negative in tubes 4 and 5. Positive reactions occur from human perspiration, urine, etc.

In carrying out the test the numerous controls must be used, all carefully marked. If the object of the test is merely to identify the suspected stain as coming from a human source, the procedure can be rapidly carried out. But if the blood is not of human origin and an opinion is desired regarding its source, it then becomes necessary to test the blood serum in turn with antisera of different specificities until a positive result is obtained. It is helpful in such cases if the circumstances give some clue as to what species blood is likely to be present.

It has been found that human blood stains dried for 10-15 yr. may still show a positive reaction. Hansemann,[365] Meyer[366] and Friedenthal have given accounts of their examinations of mummy tissue with the use of precipitating antisera. The first obtained a positive reaction with such material 4000 yr. old, the second with tissue 5000 yr. old, whereas the last named failed to get a positive reaction with mummy tissue 500 yr. old. Nuttall[367] has shown that if there is a mixture of several different bloods in solution, this does not prevent a reaction from taking place between an antiserum and its homologous blood in this test. This has been confirmed by Ziemke[368] and Smith and Glaister.[369]

Human blood stains that have been washed in water leaving only a faint color of the blood on the garment will still show the reaction.

Smith and Glaister[369] first steeped 24 hr. old and 9 wk. old human blood stains, on white cotton fabric, in cold water for as long as 5 hr. and these still yielded a precipitin reaction. In addition, an appreciable quantity of serum was also found in the steepings. They then subjected 12 wk. old stains to the action of boiling water for from 5 min. up to 1 hr. These stains still yielded a precipitin reaction following extraction with saline for a period of 24 hr. Stains 14 wk. old, however, similarly treated, failed to give a positive reaction on the application of a related antiserum. They concluded, therefore, that in case of fresh stains, boiling quickly extracts the unfixed portion of the blood serum, but in older stains the degree of fixation does not readily permit the serum to pass into solution with the water, although **prolonged** steeping in saline thereafter successfully extracts a sufficient quantity to yield a positive reaction.

If the stains have been soaked in soap powder, this contraindicates the use of this test for medicolegal purposes. Experiments on blood stains with various woods and leathers soaked with blood indicate that the test is always reliable, provided the proper dilution is made.

Alum-precipitated precipitin serum—Duncan-Taylor and Gordon:

Alum antigen:

1. Dilute 25 ml. normal serum with 80 ml. distilled water.
2. Add 90 ml. 10% potassium alum in distilled water.
3. Adjust the reaction to pH 6.5 with 5N sodium hydroxide solution and centrifuge.
4. Wash the sediment twice with 200 ml. 1:10,000 Merthiolate-saline.
5. After centrifugation dilute the final precipitate to 100 ml. with 1:10,000 Merthiolate-saline.

Ten ml. of the antigen are equivalent to 2.5 ml. serum.

Preparation of antiserum: Animals should weigh more than 4 kg. each and be in good condition.

1. Give 5 ml. amounts intramuscularly into both hind legs of the rabbit.
2. Make experimental bleedings by a single puncture of the marginal ear vein on the twentieth day after injection. If sufficiently high titer is obtained, keep the animals without food for 24 hr. and then bleed them from the carotid artery (p. 808), collecting the blood in sterile 50 ml. Rockefeller tubes.
3. Allow the blood to clot in a slanting position, loosen the clot by striking the tube against the palm of the hand, centrifuge for about 30 min., and remove the serum, observing sterile precautions. Place in a sterile graduate

Ox-blood absorption:
1. Measure the serum.
2. Add 7% by volume ox serum to the antiserum.
3. Mix and maintain at room temperature for 2 hr.; then place in a refrigerator at 5° C. overnight.
4. Centrifuge and remove the supernatant fluid.
5. Sterilize by filtration through a Seitz filter.
6. Ampule.

This alum-precipitated ox serum-absorbed antihuman precipitin serum will establish whether or not the blood stain is derived from primates, according to Gordon. **When positive with ox-absorbed serum, the stain is derived from primates.**

If any of the primates is to be excluded by a precipitin test, the antihuman precipitin serum must be prepared in an animal the species of which is to be excluded. Gradwohl[361] has reported results different from those of Taylor and Gordon in that he obtained very little difference in the reaction of chimpanzee blood with absorbed or nonabsorbed antihuman precipitin serum. The reactions were almost indistinguishable.

Reliability of "ring" test:
1. A positive reaction means the presence of human blood.
2. Primate blood (chimpanzee, gorilla, olive baboon, lemur, etc.) will give an identical reaction.
3. Absorption of antihuman precipitin serum with ox blood does not necessarily remove the quality which gives a positive reaction with the antiserum and anthropoid blood.

Flocculation test for identification of mammalian bloods—Libby photronreflectometer (Photron'er)[370]

Gemeroy et al.[370] believe that by the use of a photronreflectometer to determine exact turbidities produced by mixing an antiserum with its corresponding antigen (antihuman precipitin serum with various dilutions of human sera), a serologic correspondence curve for that antiserum can be established, and if the serum is diluted sufficiently, it no longer reacts to a significant degree with any heterologous antigen tested against it in the native state; a different correspondence curve is established for this heterologous antigen. In other words, if an antihuman precipitin serum gives positive "ring" tests with both human and rhesus monkey bloods when the serum is not diluted, the probabilities are that it will not give powerful reactions with rhesus blood when the antiserum is diluted, although it will react strongly with human serum. In the preparation of an antiserum it should therefore be possible to establish turbidity curves showing the type obtained with the antiserum in question and human serum, and the antiserum and other mammalian bloods. Gemeroy et al. have conducted numerous experiments and have produced normal titration curves of antihuman sera titrated with human, chimpanzee, and rhesus antigens. They report the serologic correspondence of the various antigens in percentage relationships with the curve produced by human serum (antigen).

To carry out this work, they use the Libby Photron'er,[370] which makes use of, or measures the amount of, scattered or reflected light that passes through a liquid. It is a delicate means of detecting finely suspended particles. A relatively small number of suspended particles reflects sufficient light to produce a significant reading on the galvanometer, and the greater the number of particles in suspension, the greater the amount of turbidity. A quantitative relationship can be established between the turbidity of a solution and the nitrogen content of a precipitate in protein antigen-antibody systems.

They use alum-precipitated serum for injection of rabbits. In the test the antiserum is serially diluted with buffered saline.

Reagents:
1. Buffered saline.
2. Sodium phosphate solution.
   Dissolve 11.876 gm. Na_2HPO_4 in distilled water and dilute to 1000 ml. with distilled water.
3. Potassium phosphate solution.
   Dissolve 9.078 gm. KH_2PO_4 in distilled water and dilute to 1000 ml. with distilled water.

Procedure:
1. Add 6 ml. of sodium phosphate solution and 4 ml. potassium phosphate solution to 50 ml. 0.85% sodium chloride solution.
2. Dilute to 100 ml. with 0.85% sodium chloride.

Serial dilutions of the serum, 1:250, 1:500, 1:1000, 1:2000, 1:4000, 1:8000, 1:16,000, 1:32,000, 1:64,000, 1:128,000, 1:256,000, and 1:512,000, are made with buffered saline. Each dilution is mixed with the antigen (serum being tested against the antiserum) and tested in the Photron'er. The sum of the turbidities noted from the complete reaction range of the antigen-antibody mixtures represents the precipitation capacity of the antiserum. Comparisons of total turbidities resulting from these summations represent the serologic correspondence of all the antigens tested with a single antiserum.

By making turbidity curves at various dilutions, using the antiserum against different

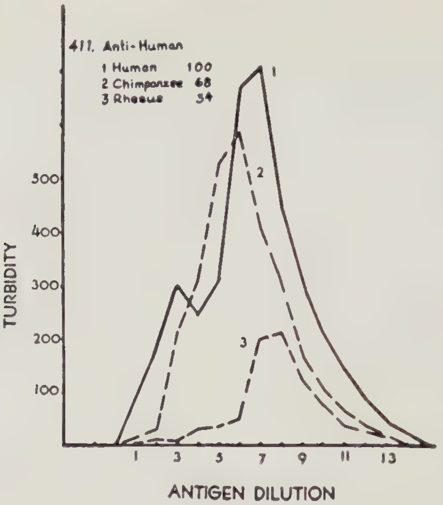

Fig. 519. Normal titration curves when an antihuman serum is titrated with human, chimpanzee, and rhesus antigens. The serologic correspondence of these antigens is recorded in percentage relationships at the top of the figure. (From Gradwohl, R. B. H.: Legal medicine, St. Louis, 1954, The C. V. Mosby Co.)

bloods, one can determine the exact dilution of the antiserum where further dilution would practically "wipe out" any reaction with certain bloods, thereby making this reaction almost species specific. It is possible that this flocculation test may find wider use in identifying specific blood when more facts have been established. For complete details, refer to Gradwohl.[361]

Tests to identify the blood group of stains

The third problem to be solved is the determination of the group of blood to which this human blood stain belongs. For practical purposes the investigation must be limited to a search for the A, B, O, and AB groups and at times for the **M** factor. Results of attempted identification of blood stains for the Rh types are disappointing. The **N** factor disappears rapidly in dried blood, but the **M** factor can be found at times, so that tests for the M-N types are likewise disappointing.

Identification of the various blood factors in a dried stain is often used in forensic medicine to prove that a certain stain did **not** come from a certain individual. If in a given case the blood group of the suspect and that of the victim are the same, no deductions can be drawn, for the stain might have come from the suspect. One must remember that a stain on clothing may be due to group receptors coming from the individual himself. Underclothing worn for several days is almost certain to contain A and B receptors if these are present in the blood and sweat.

But if the investigator obtains different results with the stain found on the suspect and the suspect's own blood, then evidently the stain was derived from a different individual. And if the stain has the same blood factors as the blood of the victim, it might or might not have been derived from the victim.

If liquid blood (unhemolyzed) is submitted, tests are run in the same manner as those used in blood grouping for transfusions. If the blood is submitted in the form of a "crust," and this blood is fairly fresh (not more than 48 hr. old) and sufficient material is submitted, the crust test may be run. Usually, however, one resorts to the agglutinin absorption test to identify blood stains.

Examination of blood crusts

A few sq.mm. of a thin crust, weighing 0.1-1 mg., are sufficient for the crust test. Thick crusts are not satisfactory since they cause the cover slip that is placed over the preparation to be raised too high.

Use a scalpel or razor blade to dislodge the crust from smooth surfaces such as glass, stone, shoe leather, etc. or to cut it from a fabric in which it is embedded.

The test cells used in the crust method should be highly sensitive, the cover glass must be manipulated to break up rouleaux in order to distinguish pseudoagglutination from true isoagglutination, and cells of group O must be used as a control against aggregation produced by other nonspecific causes.

The crust test detects isoagglutinins in the dried blood and is therefore tested against known A_1, B, and O cells.

Procedure:

1. Place 2 small, thin crusts of blood on opposite ends of a glass slide, properly labeled.

2. Near the crust on the left side place 1 or 2 drops of 2% suspension of sensitive A_1 cells, and near the crust on the right side place 1 or 2 drops of 2% suspension, in saline, of sensitive B cells.
3. Place a cover glass over each preparation so that the liquid comes in contact with the edge of the crust and no air bubbles separate the crust from the liquid.
4. Prepare another slide with another portion of the crust and add a 2% suspension of group O cells, placing a cover glass over the preparation.
5. The cover slips must be placed on the mixtures immediately, before the stain has a chance to dissolve. Avoid pressure as much as possible.
6. Wait a few minutes; then examine the preparations microscopically for signs of agglutination. While making these observations, the cover slips may be momentarily subjected to gentle pressure in order to break up any pseudoagglutination present.
7. If no results are evident, examine the slides from time to time for a period of 30 min. This will help detect weak reactions.

The group O cells added to the crust constitute the control and should show no agglutination. If these cells agglutinate, the results in the test are unreliable and must not be reported.

If the A_1 cells have been agglutinated, the stain contains anti-**A** agglutinin.

If the B cells have been agglutinated, the stain contains anti-**B** agglutinin.

The interpretation of results of the crust test are given in Table 350.

Agglutinin absorption test—Wiener

When the blood dries, the red blood cells lose their agglutinability because the free intact cells are destroyed. For this reason no direct test for agglutinogen content of the cells may be made on dried blood stains. The group-specific substances, however, retain their ability to specifically absorb their respective agglutinins. Therefore, if a serum of known titer is added to an unknown blood stain and is later retested, and if its power to agglutinate the cells has been inhibited or lost, it may be concluded that the stain contained the agglutinogen corresponding to the agglutinin in the serum. Agglutinogen A will absorb agglutinin anti-**A**, agglutinogen B will absorb agglutinin anti-**B**, and agglutinogen M will absorb agglutinin anti-**M**. Ulex anti-**H** will be absorbed by group O cells. The agglutinin absorption test is not used for identification of agglutinogen N nor for any of the agglutinogens or factors of the Rh-Hr system.

The difficulty in identifying the blood group of dried blood stains is well known to those who have attempted it. Although fresh blood can be tested for as many as 30-50 or more different blood factors, only the A-B-O groups can be determined on dried stains, and even these tests have serious limitations, as Wiener et al.[371] have pointed out. The presence of sweat, bacteria, dirt, and other interfering substances on soiled garments hampers the investigation for blood agglutinogens. One of the principal difficulties is differentiating A_2B from group B. Since isoagglutinins disappear rapidly in dried blood, sole reliance is often placed on the absorption test for blood agglutinogens, and it is frequently difficult even by this method to differentiate between blood groups A_2 and O.

Only positive reactions are significant. Negative results may indicate failure of the tests. For example, if, in the absorption test, a stain fails to neutralize anti-**A** and anti-**B** reagents, it is not permissible to identify the stain as

Table 350. *Possible results with blood crust test*

Blood crust + A_1 cells	*Blood crust + B cells*	*Blood crust + O cells*	*Agglutinin present in stain*	*Conclusions*
−	−	−	None	Either stain is group AB or there is lack of reaction due to disappearance of agglutinins
+	+	−	Anti-**A** and anti-**B**	Blood stain derived from group O individual
+	+	+	—	Test is unreliable; agglutination due to nonspecific cause
+	−	−	Anti-**A**	Blood derived from group B, or else group O with failure of reaction with group B cells
−	+	−	Anti-**B**	Blood derived from group A, or else group O with failure of reaction with group A cells

group O, since failure of absorption of the antisera might have occurred due to denaturation of the stain and not to lack of agglutinogens in it. Insufficient stain will also give negative results. If a stain neutralizes anti-**A** but not anti-**B**, a diagnosis of group A is generally justified, if one shows that the adjacent unstained material does not react with the reagents. Likewise, if the stain neutralizes anti-**B** but not anti-**A**, it presumably contains group B blood. The absorption test for group A and group B stains has an internal control, and these 2 groups can therefore usually be identified reliably, as Wiener et al.[371] have noted.

Group A_1B stains neutralize both the anti-**A** and the anti-**B** reagents. However, when one observes neutralization of both reagents, a diagnosis of group AB is not permissible because of the possibility that nonspecific absorption of the agglutinins in the reagents has been brought about by the presence of bacteria or dirt in the stain.

When the absorption method is used for grouping dried blood stains, groups A and B can usually be identified reliably but not group O and A_1B stains because of lack of an internal control. Wiener[372] used anti-**O** reagents prepared from normal bovine sera to provide an internal control in these cases since group O stains neutralize such reagents and group A_1B stains do not, but unfortunately the reactions are not always clear-cut and therefore are not suitable for medicolegal use.

The discovery that saline extracts of certain seeds give reactions similar to those of anti-**O** sera made from animal blood has been utilized in the reliable identification of blood groups of dried stains, including those that have no natural internal control. These reagents, as well as animal sera formerly called anti-**O**, have been designated anti-**H** because they do not react exclusively with group O blood cells. Anti-**H** and animal anti-**O** react in almost equal intensity with cells of groups O and A_2. Cells of group B are less strongly clumped, and A_1 and A_1B give negative or weak reactions. The reagent used by Wiener and associates is prepared from the seeds of *Ulex europaeus.*

*Anti-H reagent**

1. Grind a quantity of *Ulex europaeus* seeds in a pepper mill or, after adding saline solution, in a Waring blendor, using a microblade.
2. Dilute to approximately 10 times the original volume in a mixture of equal parts of physiologic saline and 1% sodium acid phosphate (Na_2HPO_4) solutions.
3. Allow to extract for 24 hr. in a refrigerator.
4. Remove the suspended particles by centrifugation.
5. Pass the supernatant opalescent extract through a Seitz filter to clarify it and to remove bacterial contaminants.
6. Store in a refrigerator until needed.

Standardize by direct titration against human red cells of A-B-O groups and by testing the capacity of saliva from secretors to inhibit its reaction with group O test cells. Table 351 shows results of a sample titration of an anti-**H** reagent against known red cells of various groups at refrigerator temperature. It will be seen that 2 different group O cells gave titers of 8 units and 16 units, respectively, or an average of about 12 units. Group A_2 cells showed approximately the same titer, whereas group B cells reacted to a considerably lower titer and groups A_1 and A_1B showed the weakest reactions.

Dilute the reagent to a titer of not less than 5 and not more than 10 units for group O cells.

Inhibition test—Wiener

Carry out the inhibition test on saliva with saliva from known secretors of each blood group and also from known nonsecretors, using the method on pp. 1543ff with saliva diluted 1:1, 1:4, 1:16, 1:64, and 1:256. The results of the tests are expressed as inhibitor titer units (Table 352). Anti-**H** reagents that give reactions similar to those in Table 351 are satisfactory to use in tests on dried blood stains.

Agglutinin absorption tests for identification of dried blood stains

Preparation of antiserum: Dilute the anti-**A**, anti-**B**, and anti-**H** sera to yield reagents with a titer of between 5 and 10 units. This means that each antiserum must have been titrated and its titer determined before use. To titrate, dilute the antiserum 1:10 by adding 1 drop of antiserum to 9 drops of saline, and then proceed with serial dilutions: 1:10, 1:20, 1:40, 1:80, etc., using the method of diluting given on p. 1476. To each diluted serum (1 drop in its respective tube) add 1 drop of cell suspension containing the agglutinogen corresponding to the agglutinin in the antiserum (A_1 cells to anti-**A**, B cells to anti-**B**, etc.). These tests may be carried out in tubes, or they may be made on slides. The tubes must remain at room temperature for at least 1 hr. before being read. The slides may be read microscopically at the end of 8-10 min. shaking on a rotator. The reciprocal of the weakest dilution of the antiserum that gives a definite reaction is the titer. If the titer of the serum is, for example, 640, then dilute the antiserum 1 part serum plus 63 parts saline to obtain a final titer of 10 units, or 1 plus 127 for 5 units, etc. The antiserum is diluted until a titer of 5-10 units is obtained.

Use the same A, B, and O cells in the agglutinin absorption tests as are used in the titration. The titration thus becomes a control over the cells.

---

*The prepared anti-**H** reagent can be purchased from Certified Blood Donor Service, Inc., Jamaica, L. I., N. Y.

Table 351. *Titration of anti-H reagent (Ulex europaeus) against various human red cells**

Specimen No.	Blood group	Undiluted	Reactions with seed extract diluted 1:2	1:4	1:8	1:16	1:32	Titer
1	O	++±	++	++	+	–	–	8
2	A_2	++±	++±	++	+±	Trace	–	12
3	B	+++	+±	+	Trace	–	–	4
4	O	+++	+++	++±	++	+	–	16
5	A_1B	+	Trace	Trace	–	–	–	1
6	A_1B	Trace	–	–	–	–	–	0
7	A_1	Trace	–	–	–	–	–	0
8	A_1	+±	+±	Trace	–	–	–	3
9	A_2	+++	++±	++	+±	Trace	–	12

*From Wiener, A. S.: Lab. Digest, Feb., 1959.

The strength of the reaction is indicated by the number of plus signs as follows:

+++, one large clump.
++±, several large clumps.
++, many finer clumps.
+±, very fine clumps barely visible to the naked eye.
+, distinct clumping but visible only under the microscope.
±, clumps containing only a few cells each.
–, no clumping.

The titer is the reciprocal of the highest dilution giving a distinct (1 plus) reaction.

Table 352. *Sample protocol of inhibition tests with anti-H reagents against saliva—samples from different individuals**

Specimen No.	Blood group	Secretor status	Reaction with group O cells of mixture of anti-H reagent and boiled saliva diluted: Undiluted	1:4	1:16	1:64	1:256	Inhibition titer
1	O	Secretor	–	–	–	–	+±	64
2	O	Secretor	–	–	–	Trace	++	32
3	A_1	Secretor	–	–	–	++	++±	16
4	O	Nonsecretor	++	++±	++±	+++	+++	0
5	B	Nonsecretor	++	++±	++±	++±	+++	0
6	A_1	Secretor	–	–	++	++±	++±	4
7	A_1	Nonsecretor	+++	+++	+++	+++	+++	0
8	O	Secretor	–	–	Trace	++	++	8
9	A_1	Secretor	–	Trace	++	++	++±	2
10	B	Secretor	–	–	++	++±	+++	4
11	A_1B	Secretor	–	–	Trace	++	++±	8
Reference standards	Commercial solution of blood group substances		–	–	–	–	+±	64
	Nonsecretor saliva		++±	+++	+++	+++	+++	0

*From Wiener, A. S.: Lab. Digest, Feb., 1959.

Procedure:

1. If the blood is in the form of a crust or powder, and sufficient specimen is obtainable, grind it into very fine powder, and add 10 mg. portions to 0.1 ml. of each testing serum (anti-**A**, anti-**B**, anti-**H**) in small tubes.
2. Mix thoroughly with the aid of a stirring rod and then allow it to stand in a refrigerator overnight to complete the absorption. Centrifuge and test the supernatant dark-red fluid for its agglutinin content. (See examination of blood crusts.)
3. If the material has been absorbed, as it will be on clothing, paper, and other articles, cut out portions of the stained and also the unstained areas, of approximately equal size, and use these for the tests and controls. It is essential that controls be run, using material from the unstained portion of material, since this at times contains one or both of the blood group receptors from sweat, urine, or animal material. If the tests for the receptors in the unstained material are positive, the results obtained in tests on the stained area cannot be relied upon.
4. Use glassware and equipment that contain no blood or body secretions. Avoid touching any of the material with the bare hand. Always mark the portion of specimen from which the stain and the unstained areas have been cut, using an indelible pencil, for possible later identification in court.
5. Cut portions of both stained and unstained material of approximately equal size and place in separate small (7 mm. inside diameter) tubes. Prepare 8 tubes for the duplicate sets of tests of the stain (labeled 1, 2, 3, 4,-test) and 4 tubes for the controls on the unstained

portion (labeled 1, 2, 3, 4,-controls). Controls using known dried blood stains containing, respectively, groups A_1, A_2, B, O, and type M blood are described below. These last need not be made each time a test is run, but such controls should be made often enough to check the antisera and the technic of the individual performing the tests.

6. Add enough anti-**A** serum to each tube 1 of the test and control tubes to make the cloth sopping wet; anti-**B** serum to each tube 2; anti-**H** Ulex to each tube 3; and anti-**M** serum to each tube 4. These are the antisera that have previously been diluted to a titer of 10.
7. Prod the contents of each tube with a glass rod to make the cloth absorb the serum. There should be no run-off of the serum when the tube is tilted. A different glass rod should be used for each tube.
8. Refrigerate the duplicate 4 tests and the 4 control tubes overnight.
9. The following day aspirate the serum in each tube by pressing against the cloth with a capillary pipet with a rubber bulb, using a different capillary pipet for each tube, and place the aspirated serum in small tubes properly labeled.
10. To each tube 1 add 1 drop of saline-suspended group A_2 cells (2% concentration).
11. To each tube 2 add 1 drop of saline-suspended group B cells.
12. To each tube 3 add 1 drop of saline-suspended group O cells.
13. To each tube 4 add 1 drop of saline-suspended type M cells.
14. Mix each tube thoroughly and place the tests containing anti-**H** in a refrigerator for 2 hr. Leave the other tests (A, B, M) at room temperature. The A, B, M tests may be made in welled slides.
15. Read for agglutination or lack of agglutination.
16. The control tubes should all show positive reactions. If they do not, the test is unreliable. See Table 354.

The results possible with this test are given in Table 353.

Controls using known dried blood stains: The dried stains are prepared by adding blood of known groups to different pieces of cloth and keeping them refrigerated until ready for use. The cloths are usable as controls for about 2 mo. Each stain is tested with anti-**A**, anti-**B**, and anti-**H** diluted serum, as in the tests. The technic is carried out as outlined above for the unknown stain: a segment of the cloth is put in each of a number of tubes, the proper antiserum is added until the cloth is sopping wet, and the cloth is prodded until it soaks up all the antiserum; then it is left at refrigerator temperature overnight, and the next day tests are made on the serum, using various cells. See Table 354.

When there are large stains not in crust form, the following method may be used. The crust technic (p. 1618) cannot be used unless the stain is fresh, but the **following method may be used on large stains.**

1. Extract the stain to a dark color, using 30-40 drops or more of saline.
2. Express the fluid from the cloth.
3. Centrifuge hard. This is an agglutinin extract.
4. Place 5-10 drops of the fluid in each of 3 small tubes.
5. Add A_2 cells to the first tube, B cells to the second, and O cells to the third.
6. Refrigerate for 10 min.
7. Centrifuge in ice cups.
8. Remove the supernatant fluid and add a drop of saline to each sediment before reading. Read microscopically; see Table 357 and then verify with the Ulex method.

This last method is simply a screening procedure and is not used for conclusive results.

It must be kept in mind that these are methods for detecting indirectly the presence of certain agglutinogens in a blood stain and are not direct tests for blood groups as entities. It may be stated in a report that a stain contains a certain agglutinogen but not that a certain agglutinogen is absent.

Sources of errors in interpreting results of blood grouping tests of dried stains[373, 374]

Numerous errors are possible in interpreting the results in the blood grouping tests of dried blood stains.

1. If the preliminary test for determining

Table 353. *Results possible in agglutinin absorption tests*

*Anti-**A** serum* + *cloth*	*Anti-**B** serum* + *cloth*	*Anti-**H** Ulex* + *cloth*	*Anti-**M** serum* + *cloth*	*All absorbed overnight*
Cells added as follows				
A_2 cells	*B cells*	*O cells*	*M cells*	*Blood group of stain*
+	+	−		Group O
−	+	+ or −		Group A (A_2 cells react with anti-**H**)
+	−	+		Group B
−	−	+		Group AB
			−	**M** factor present in stain
			+	**M** factor not present in stain

Table 354. *Preparation of controls on stained cloth*

A_1 blood stained cloth			A_2 blood stained cloth			B blood stained cloth			O blood stained cloth		
+			+			+			+		
anti-**A**	anti-**B**	anti-**H**	anti-**A**	anti-**B**	anti-**H**	anti-**A**	anti-**B**	anti-**H**	anti-**A**	anti-**B**	anti-**H**
diluted serum			diluted serum			diluted serum			diluted serum		

Add A_1 cells to the first tube containing anti-A serum and A_2 cells to each remaining tube containing anti-A serum, B cells to each tube containing anti-**B** serum, and O cells to each tube containing anti-**H** Ulex. Mix thoroughly and allow to stand for 2 hr. before reading. The results possible are given in Table 355.

Table 355. *Results possible in controls on known dried blood stains**

			Results
A_1 stained cloth +			
(1) anti-**A** serum	(2) anti-**B** serum	(3) anti-**H** Ulex	
The antisera absorbed overnight; then cells added:			A_1 cells negative
A_1 cells	B cells	O cells	All others positive
A_2 stained cloth +			
(1) anti-**A** serum	(2) anti-**B** serum	(3) anti-**H** Ulex	A_2 cells negative
The antisera absorbed overnight; then cells added:			B cells positive
A_2 cells	B cells	O cells	O cells negative
B stained cloth +			
(1) anti-**A** serum	(2) anti-**B** serum	(3) anti-**H** Ulex	
The antisera absorbed overnight; then cells added:			B cells negative
A_2 cells	B cells	O cells	All others positive
O stained cloth +			
(1) anti-**A** serum	(2) anti-**B** serum	(3) anti-**H** Ulex	
The antisera absorbed overnight; then cells added:			O cells negative
A_2 cells	B cells	O cells	All others positive

*If desired, tubes may be prepared for groups A_1B and A_2B dried blood and for type M.

Table 356. *Controls on unstained area**

Unstained material	Unstained material	Unstained material
+	+	+
anti-**A** serum	anti-**B** serum	anti-**H** Ulex
Absorbed in tubes overnight in a refrigerator		
+	+	+
A_2 cells	B cells	O cells

*All reactions should be positive unless some material present in the cloth absorbs the antibody. (The antisera have been diluted to a titer of 10 units before use.)

Table 357. *Results possible with extracts of large stains*

Stain extract + *A_1 cells*	*Stain extract* + *B cells*	*Stain extract* + *O cells*	*Blood group of stain*
+	+	−	Group O
−	+	−	Group A, or O with anti-**B** deteriorated
+	−	−	Group B, or O with anti-**A** deteriorated
−	−	−	Inconclusive

the titer of the antiserum used is satisfactory, a +++ or ++ reaction is obtained against the known cells (which are to be used in retesting the serum after absorption), and if in the actual test the control from the unstained area gives only a + or ± reaction, then something containing the group substance—sweat, saliva, urine, etc.—is present, and the activity of the antiserum has been inhibited. Under these circumstances it is not advisable to base any conclusions concerning the group of the stain on the results of the absorption test. If possible, another test of unstained material taken from an area closer to the stain should be made to determine if the interfering material is localized or is to be found all over.

2. If in the preliminary test to determine the titer of the serum a +++ or a ++ reaction is obtained against the known cells which are to be used in retesting the serum after absorption, and if in the actual test after the serum has been submitted to absorption by a known or unknown stain a ± result is obtained, it indicates that the group-specific substance has reduced the reactivity of the serum.

There are 3 possible explanations: (a) the stain is old or has been exposed to conditions unfavorable for the preservation of the group substance; (b) the amount of blood in the stain is insufficient in quantity; (c) the serum used was of too high a titer. In the case of controls, the first 2 reasons may be eliminated by using freshly prepared, adequate samples.

The last possibility may be eliminated by performing another test on another portion of the stain, using serum of lower titer, i.e., by diluting the serum originally used with an equal volume of saline. Clear-cut negative reactions are required in the controls, using blood stains of known type, if the group of a stain is to be based on the absorption test.

3. If the test of unstained material, known group O, and the corresponding known stain in the controls give +++ or ++ results, a ± result with the unknown indicates that the reactivity of the serum has been inhibited. This reduction in titer is attributable to group-specific substances present in the unknown stain.

4. It must be kept in mind that with an unknown stain a positive result may be obtained because of disappearanec of the group factor due to age, exposure to undesirable physical conditions, or other causes. An insufficient quantity of stained material may also account for these results. Identification of an unknown stain as group O by the absorption method then is provisional (except when the reagent anti-**H** Ulex is used, p. 1620). Group O stains may be identified absolutely by testing for and finding both agglutinins present in the stain, as in the crust technic.

5. The $\mathbf{A}_2$ factor is considerably weaker than the $\mathbf{A}_1$ so that a ± reaction or a marked reduction in the titer of the anti-**A** serum (i.e., from a +++ to a + reaction) indicates that only a partial removal of anti-**A** agglutinin has been achieved. Retesting the stain with serum of lower titer will be useful in confirming the A_2 diagnosis.

In the grouping of dried stains, to a degree far greater than elsewhere in forensic blood tests, technic, experience, and the condition of the stain—exposure to elements, age, amount, and where found—are of paramount importance. The absence of group-specific factors in the stain does not necessarily mean that the blood is that of group O. In particular, the possibility of its being an A_2 must be considered. Similarly, if a stain is found to contain the B agglutinogen, consideration must also be given to the possibility of its being an A_2B blood group.

The use of the reagent, anti-**H** Ulex, helps resolve some of these difficulties.

Chapter 83

Medicolegal aspects of blood transfusion

ADDINE G. ERSKINE

This section cannot be closed before considerable attention is paid to an important report by the Committee on Medicolegal Problems of the American Medical Association.[375] This Committee was composed of Alexander S. Wiener, Chairman, and David N. W. Grant, Lester J. Unger, and William G. Workman. They represent a group of outstanding serologists with a vast experience in this field. The following remarks are based largely upon this Report, and although at the time of the report the material is authoritative, one cannot predict from the rapid strides that are now being made whether or not in the future they will necessarily be modified.

The report states that approximately one death in 1000-3000 transfusions has been reported. This makes blood transfusion as important a cause of death as appendicitis or anesthesia.

Errors are frequently made during emergency periods when the regular technician is not at hand, where a certain number of errors are inevitable. They advise that all hospitals should carry liability insurance and that medical schools should devote more attention to blood grouping in their curricula.

Unfavorable results have occurred because blood grouping sera have been inadvertently interchanged and also when 2 patients require transfusions at the same time and the bottles of blood intended for them have been interchanged. In a few cases errors have resulted because the wrong pilot tube was affixed to a bottle of blood. Most transfusion accidents due to mixing up of bloods can be traced to a failure to read labels. Colored grouping sera and labels have the disadvantage that the technician has a tendency to rely on his memory of the colors instead of reading the labels on the bottle of blood.

If a patient dies as a result of a transfusion of blood of an incorrect group or suffers prolonged illness due to injury to his kidneys, the patient's family or the patient himself, if he survives, may be entitled to damages.

One of the commonest causes of hemolytic reactions is the transfusion of blood of an incompatible blood group.

Anti-**A** and anti-**B** isoantibodies in human sera are generally of low titer and avidity, and, if so, an initial transfusion of group A blood to a group O individual might be uneventful or produce only a slight reaction. As a result of such a transfusion of incompatible blood, the isoantibody titer will rise, so that if the error is repeated a fatal reaction could result.

The severity of the reaction in strongly sensitized individuals depends on the amount of blood transfused, fatalities in adults being infrequent with quantities of blood less than 300 ml. Therefore, if this portion of each unit of blood is given slowly and the patient is watched closely during that time for untoward symptoms, it may be possible to interrupt the transfusion in time should a reaction develop. This is unlikely to be detected in patients under anesthesia.

SUBGROUPS OF A

The A_2 agglutinogen is characterized by its weaker reaction with anti-A serum. The reactions are especially weak in the presence of agglutinogen B, so that blood of subgroup A_2B has often been incorrectly classified as group B. The low avidity of most anti-**A** sera for A_2B blood which is responsible for errors in blood grouping also protects the patient receiving such incompatible blood from any dangerous reaction.

Rh FACTOR

For blood transfusion practice it is sufficient to limit the tests on patients to only one of the Rh-Hr factors, $\mathbf{Rh_0}$, the factor that corresponds to the one originally discovered with the aid of anti-rhesus immune animal sera. Generally, the initial transfusion of Rh-positive blood into an Rh-negative recipient is symptomless. In individuals who have been previously sensitized as a result of an injection of Rh-positive blood or a pregnancy with an Rh-positive fetus, even the first transfusion of Rh-positive blood can cause a severe reaction.

By carrying out cross-matching tests by the modern technics, namely, the saline, the conglutination, and the antiglobulin methods, incompatibilities with respect to the other factors of the Rh-Hr systems can be detected should isosensitization be present.

Persons with blood possessing an $\mathbf{Rh_0}$ variant may be incorrectly classified as Rh negative. A transfusion of blood containing an $\mathbf{Rh_0}$ variant into an Rh-negative recipient can give rise to isosensitization.

Under certain conditions Rh-negative blood may seem to react as Rh-positive. For instance, if bloods from pregnant women are tested by the slide technic, rouleaux formation, which often occurs in pregnancy, may be mistaken for clumping. In acquired hemolytic anemia the red blood cells may be coated with autoantibodies, which may be responsible for clumping in tests carried out by the conglutination method.

In most cases of transfusion reactions in which Rh-positive blood is transfused to Rh-negative individuals, the error can be traced to some laboratory error rather than to a peculiarity in the blood itself. A hemolytic reaction occurring after a transfusion of Rh-positive blood into an Rh-negative patient is usually evidence of negligence.

The selection of donors is no longer a simple process. Any blood bank and hospital where blood transfusions are carried out should have a specially trained technician to handle the work under qualified medical supervision. The dangers of transfusion have been summarized by Unger[376] and Wiener.[377]

UNIVERSAL DONORS

Although transfusion of group A blood into a group O recipient may cause a serious reaction, the transfusion of group O blood into a group A recipient is generally harmless. In the former case the small volume of incompatible donor's cells combines with the isoantibodies present in a much larger volume of the recipient's plasma, whereas in the latter instance a limited amount of incompatible donor's plasma is diluted in the recipient's circulation and absorbed by the great mass of the patient's red blood cells. Blood containing incompatible agglutinins is much less dangerous than blood containing incompatible agglutinogens.

Transfusions of group O blood into individuals of other groups may be dangerous. Hemolysis may follow such transfusions when the donor has isoagglutinins of high titer in his serum. Although injection of high-titered group O blood into individuals not belonging to group O may produce an acute hemolytic reaction, the symptoms are usually somewhat different from those that occur when incompatible red blood cells are injected. The use of group O blood as "universal donor" blood should be restricted to emergencies where there is not enough time to determine the group of the recipient reliably.

If it is not always possible to carry out all the tests necessary in cases of emergency, i.e., tests relative to the Rh factor, preferably low-titer group O Rh-negative blood should be used for transfusions, especially in women during the childbearing period. One must balance the danger of hemorrhage against the danger of isosensitization. A suitable entry should be made on the patient's chart to justify the course of action taken.

The transfusion of Rh-negative blood of the proper blood group into an Rh-positive recipient is almost always safe. Careful cross-matching tests must be done to detect incompatibilities aside from those due to the $\mathbf{Rh_0}$ factor (Rh subtypes, **hr′**, etc.).

OTHER BLOOD GROUPS

The sera of certain rare individuals contain irregular isoantibodies for blood group factors other than **A** and **B**, for instance, anti-**M**, anti-**P**, and anti-**Lewis**. In addition, immune antibodies to blood factors besides Rh-Hr may be formed as a result of isosensitization, notably anti-**M**, anti-**Kell**, and anti-**Duffy**. This may cause serious hemolytic reactions.

The Committee does not believe that reactions caused by sensitizations to one of the other blood factors can properly be a cause of action for a lawsuit, provided that the physician and hospital have exercised ordinary care to protect the welfare of the patient.

DAMAGED BLOOD

Blood damaged during storage may break down rapidly in the patient's circulation and give rise to hemoglobin nephrosis. No blood should be used if, after inspection, hemolysis is suspected.

Red blood cells are sensitive to freezing and overheating. Dry ice must not be used. No attempt should be made to warm blood prior to or during its administration. Blood stored too long or improperly can give rise to transfusion hemolysis. The National Institutes of Health permit an expiration date of 21 days for blood stored in citric acid-dextrose solution.

Blood should be kept cold from the moment it is collected until it is used. It should be stored in a refrigerator set preferably at 4°-6° C. The temperature of the refrigerator must be recorded at least once a day.

TRANSMISSION OF DISEASE

One of the most serious dangers is transmitting homologous serum hepatitis to the patient. This may occur once among 200 patients receiving transfusions and may be responsible for the patient's death once among 6000 transfusions. The danger is graver following plasma transfusion.

Homologous serum hepatitis was observed during World War II as a result of the extensive use of pooled dried plasma when treating battle casualties. Pooling increases the hazard of homologous serum hepatitis, because the presence of the virus in a single sample of blood plasma will serve to contaminate the entire pool. The most important difference between homologous serum hepatitis and infectious hepatitis or catarrhal jaundice is the mode of transmission. There is no simple test by which the carrier of the virus can be recognized, and the presence of the virus in the plasma pool can be detected only by testing it on human volunteers.

The National Institutes of Health have required that pooled plasma be irradiated before distribution, and this ruling is still in effect. Recent observations, however, indicate that such irradiation may not always be effective. Irradiation is not applicable to whole blood. The problem of homologous serum hepatitis has not yet been solved.

Syphilis: Transmission of syphilis by transfusion is rarely heard of any longer.* This is mainly due to the development of more sensitive tests for the detection of the disease in prospective donors, whereas another effective factor in preventing the disease is the widespread use of bank blood. According to this report: "Since the agent of this disease cannot live more than a few days outside the body, even at refrigerator temperature, the danger of transmitting syphilis is virtually nonexistent when blood or plasma aged for at least three days is used for transfusions." **No individual with positive serologic tests for syphilis or positive history of the disease should be used as a donor.**

Malaria: Transmission of malaria by transfusion is a difficult problem in regions where the disease is endemic, but until recently it was not an important problem in the United States. For obtaining fresh blood for transfusion, individuals who have had malaria or who have received suppressive therapy for malaria while residing in a malarious region should not be used as donors for a period of at least 2 yr. following the last proved attack or discontinuance of suppressive therapy. The *Plasmodium malariae* can survive in a refrigerator for as long as 5 days, so that bank blood less than 5 days old might be infectious.

Other infectious diseases: Other infectious diseases, such as measles and influenza, have been transmitted by blood transfusion when the donor was in the prodromal stage of the disease at the time his blood was used for transfusion. All prospective donors should be examined to detect the presence of infectious disease.

CONTAMINATED BLOOD

Bacteriologic surveys have shown that as many as 5-10% of bank blood specimens are contaminated with bacteria. No harm results usually because the majority of microorganisms present are gram-positive bacteria relatively low in pyrogenicity, do not grow at refrigerator temperature, and are generally nonpathogenic. Some bacteria grow at refrigerator temperature.* Transfusion of such blood has resulted in death of the patient. At this time it is not considered advisable to add any antiseptic to blood in an attempt to prevent bacterial growth. Prime reliance must be placed on stringent aseptic technic when collecting and storing blood.

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*Elsewhere in this section (p. 1568) will be found Gradwohl's views on this subject. His views are based upon experiences, not with blood bank blood, but with fresh blood from individual donors.

ACCIDENTS FOLLOWING TRANSFUSION

Embolism: During storage of blood or plasma, precipitation of fibrin gradually occurs, and if such blood or plasma is transfused unfiltered, multiple embolization into the lung bed may occur, or into the viscera or brain if the recipient has a cardiac septal defect. All bank blood and stored blood should be filtered during transfusion.

Rapid transfusion of blood by pumping air under pressure into the blood reservoir has caused a number of deaths from air embolism. This type of transfusion should be condemned.

Allergic reactions: About 1% of blood transfusions are followed by reactions of an allergic type. These reactions are mild and consist merely of urticaria. Angioneurotic edema is a more serious allergic manifestation which may be dangerous if it involves the larynx. It is best not to use allergic individuals for blood donors.

Pyrogenic reactions: Pyrogenic reactions have been caused by growth of bacteria in the water used for preparing solutions or in moisture adherent to the apparatus before they are sterilized.

Methods have been developed for preparing pyrogen-free solutions and apparatus, which are readily available commercially. (See pp. 1593ff on pyrogenicity.) Therefore, pyrogenic reactions should no longer be an important problem.

Overloading: Too rapid transfusion or transfusion of too much blood may cause death from congestive cardiac failure or cerebral hemorrhage. A certain number of deaths from too rapid transfusions still occur, especially when transfusions are given to infants.

Citrate reactions: The sodium citrate which is introduced during transfusion is rapidly metabolized and excreted. The amount of citrate administered with 500 ml. blood is harmless to the adult. When massive transfusions are given in a short period of time, sodium citrate may accumulate in the blood stream, so that signs of hypocalcemia may result, namely, a drop in blood pressure and tetany. This may happen when adults with massive hemorrhage require more than 1 L. of blood. If 10 ml. 10% solution of calcium gluconate are injected slowly intravenously after every 1000 ml. citrated blood, hypocalcemia may be prevented. When performing exchange transfusions on erythroblastotic babies, some transfusionists inject 1 ml. 10% calcium gluconate after every 100 ml. citrated blood; others meet the problem by removing part of the citrated plasma from the blood to be used for the transfusion.

INTRAMEDULLARY TRANSFUSION

In adults, bone marrow transfusion is made through the sternum. In infants and small children in whom the sternum is too thin, the tibial route is used. The Committee believes that the intramedullary route for ordinary transfusions is not recommended because of the danger of fat embolism, especially in children. The procedure has resulted in osteomyelitis and fractures of the tibia. Intramedullary transfusion has its main application in instances of extensive burns or in patients with skin diseases, which makes intravenous therapy difficult.

OTHER ROUTES

Transfusion into the longitudinal sinus in inexperienced hands may be dangerous because the blood may be injected extradurally. Nobody any longer advocates the use of intraperitoneal or penile routes.

HOW TO PROTECT THE BLOOD DONOR

If a donor gives blood too frequently, there is likelihood of secondary anemia, a danger greater in women than in men. Donors should not be permitted to donate a pint of blood more often than every 6 wk. or until the blood attains a hemoglobin concentration of at least 12.5 gm./100 ml., or a specific gravity of 1.053 or higher.

A sterile lancet, preferably expendable, should be used when obtaining blood for measuring hemoglobin concentration, to avoid transmission of hepatitis virus.

Fainting: Donors often faint when blood is taken or when they see other blood taken. Aside from the psychic aspect, the cause is sudden reduction in blood volume. The in-

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*Stevens et al.[378] reported a fatal transfusion reaction due to cold growing *Pseudomonas* sp. in stored blood.

cidence of fainting can be reduced by screening out unsuitable donors by physical examination to exclude those with low blood pressure or a low hemoglobin level or with a small body size. A donor who has been without food for a long time or who makes his donation after coming off a night shift may be a candidate for fainting. He should be given a light snack before taking his blood. After the donation the donor should be kept in a supine position until he has apparently recovered. The nervous donor should have his attention diverted from the procedure by pleasant conversation. The room should be light and dry, and smoking should not be permitted in it or in the resuscitation room. On hot days the donors may be given some isotonic sodium chloride solution before the bleeding. If the donor faints, keep the head low, loosen the clothing, and provide with fresh air. In rare cases intravenous infusions of sodium chloride may be required.

Syncope occurs sometimes after the donor has left the hospital. He may fall and lacerate his scalp or even fracture his skull or become involved in a serious accident if he falls in the street. Donors involved in hazardous occupations should be advised not to return to work for at least 12 hr.

Thrombosis and infections: In rare cases thrombophlebitis or abscess formation has occurred at the site of the puncture. It is wise for the blood bank physicians to protect themselves with suitable insurance to prevent a lawsuit.

Donation of a pint of blood by a healthy adult is safe.

PREPARATION OF BLOOD DIAGNOSTIC SERA

Blood grouping tests are possible only when potent and specific diagnostic sera are available.

Men are used more often than women for the preparation of diagnostic sera. No Rh-negative woman should be immunized with Rh-positive blood to prepare anti-Rh sera if there is any chance of her becoming pregnant later. Similarly immunization of a group A woman against agglutinogen B or vice versa might cause obstetric complications. More common sera, such as anti-**A**, anti-**B**, and anti-$\mathbf{Rh}_0$, can be prepared by immunization of male volunteers, but this technic is not applicable to weaker factors such as **rh′**, **rh″**, **hr″**, **Kell**, and **Duffy**. To prepare anti-**A** and anti-**B** sera, a number of different immunization procedures are used.[379] Intravenous injection of blood group specific substances prepared from horse and pig stomach carries the possibility of anaphylactoid reactions in allergic subjects. This can be avoided by using deproteinized preparations and by administering them intramuscularly instead of intravenously. Some workers also use human plasma to prepare anti-**A** and anti-**B** sera. Some subjects complain of pain along the course of the vein during the injection or have a mild rise in temperature later. The former can be avoided by diluting the plasma with sterile physiologic saline solution and injecting it more slowly, the latter by having the donor take 5 gr. or 0.32 gm. acetylsalicylic acid at the time of the injection or by reducing the dosage. Look out for the danger of homologous serum hepatitis.

Before injecting blood into any volunteer, explain the purpose. The sensitized Rh donor should be given a suitable identification card. He should be warned that only Rh-negative blood should be used for any transfusion he may require in the future. A signed release should be secured from the donor.

Occasionally, volunteers immunized against the Rh factor have developed antibodies against other antigens, most frequently agglutinogen M. This can be avoided by testing all subjects for the M-N types and using only type N, Rh-positive blood for Rh-negative donors of type N.

Physicians are urged to enlist the cooperation of patients whose plasma contains rare blood group antibodies so that their blood can be used for the preparation of diagnostic sera. Insurance companies specialized in malpractice insurance can aid the work by providing riders on policies of physicians specializing in this field.

Every hospital should have one or more qualified individuals responsible for the transfusions performed there. These should see that the residents are properly trained to perform transfusions and should be available to advise and assist them when problems of unusual difficulty arise. When nurses or technicians are used in the blood banks for blood withdrawal, this should be under the direct supervision of a qualified physician.

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PART V

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Section One

TISSUE CUTTING AND STAINING

The purpose of preparing stained sections of tissue is to present the slides to the pathologist for microscopic examination. These slides become permanent records of the living patient and of autopsies performed on the cadaver. It is of the utmost importance that tissues be properly handled from the time of removal from the body to presentation of the slides to the pathologist. Proper fixation must be carried out at once to prevent cellular changes and shrinkage. Infiltration of the tissue, embedding, sectioning, mounting, and staining all require critical technical manipulation. The sections must be cut as thin as possible, there must be no shrinkage, no folding, and no bubbles, and the differentiation of the cells and cellular nuclei must be distinct. As a rule, there should be but a single layer of cells in the section. The clarification of sections to permit passage of light through them is of vital importance. Each step in the preparation of tissue sections for pathologic examination must be thoroughly mastered, since the correct reading of the tissue depends to some extent on the final product submitted for examination. Strict adherence to technic is essential until the various processes are completely mastered, after which the preparation of tissue sections becomes more an art than a science.

The handling and examination of pathologic tissues have constituted for many years most important steps in the development of the hospital laboratory. Every piece of tissue removed at the operating table must be submitted to a pathologic investigation both macroscopic and microscopic. One of 3 methods is generally used: the paraffin, celloidin, or frozen section technic. The celloidin method is often used only in specialized work. The most commonly employed method is the paraffin technic. Frozen sections are made in some institutions as a routine procedure and in others only in emergencies.

Chapter 84

Tissue fixation

ARAM A. KRAJIAN

The term **tissue fixation** means the preservation of the normal structure of a tissue as closely as possible to its condition during life. It is necessary, therefore, to fix and harden tissues intended for microscopic examination as soon as possible after removal from the body either during life or after death. The immediate fixation of the tissue not only preserves the cells and intracellular substances but also prevents changes that take place after death because of the putrefactive action of bacteria.

The principal purpose of the fixation is to cause coagulation or precipitation of protoplasmic substances, which renders the cells and tissue elements resistant to such further chemical effects to which they are subjected before microscopic sections are prepared. The most commonly used fixative is a solution of formaldehyde, a gas, which is soluble in water to the extent of 40% by weight. It is called **formalin.** It has the advantages of penetration and fixation with great speed, thus enabling one to cut and stain frozen sections as well as paraffin sections. The best strength is a 10% solution.

Some fixatives, such as **Zenker fluid,** act as mordants, which cause a particular staining reaction. Other fixatives, such as alcohol or isopropanol, have a hardening effect. Zenker solution is very reliable because it preserves nuclear structure, bacteria, and fibrils of all kinds.

Stowell[1] has made a distinct contribution to the effect on tissue volume of fixation, dehydration, and embedding. He found that all fixatives showed specific differences. Susa and Bouin fixatives gave the best general results and neutral formalin mixtures, especially pyridine-formalin, the poorest results. Isotonic saline is superior to distilled water as a formalin diluent because it reduces tissue swelling during formalin fixation. Reagents producing marked decreases in tissue volume render such tissues less susceptible to shrinkage during subsequent procedures. Shrinkage of tissue during dehydration and infiltration with hot paraffin may exceed that produced by fixation alone. Excessive heat causes tissue distortion and shrinkage. Infiltration with hot paraffin causes considerable shrinkage; with hot nitrocellulose, less shrinkage; and with cold nitrocellulose, the least shrinkage.

Alcohol hardens and dehydrates tissue at the same time. Tissues kept in alcohol, however, cannot be immediately used for frozen section work. Alcohol has a tendency to produce too great hardness in tissue, rendering it difficult for sectioning purposes. It does not penetrate as well as formaldehyde so that the tissues must be cut in very small blocks.

In general, all tissue for fixation must be of a size sufficient for penetration. The **volume of the fixative** solution must be at least 25 times that of the tissue. The smaller the tissue, the smaller the receptacle need be to obtain this 25:1 ratio.

When formaldehyde is used as a fixative, the tissues are placed in 15% formalin for the first 24 hr.; then they are properly trimmed into square or rectangular blocks, placed in 10% solution, and permitted to remain there. When tissues are **fixed in Zenker solution** or other chrome salt solutions, the solution need not be changed.

To fix eye specimens after enucleation, inject the tissue with 10% formalin and place the tissue in a similar solution for 10 days before trimming for microscopic section.

Tissues previously fixed in alcohol can be washed under running tap water for 15 min. and then placed in 10% formalin for 24 hr., after which frozen sections may be cut.

FORMULAS FOR TISSUE FIXATIVES OR HARDENERS

The fixatives selected depends on the nature of the pathologic lesion in the tissue. All types of fixatives are not compatible with all staining methods; some fixing reagents act as mordants for a particular staining process, whereas others inhibit certain stains. In general, Bouin, Zenker, mercuric bichloride–formaldehyde combination, mercuric chloride, and Carnoy fluid allow most of the staining, whereas formaldehyde prevents carmine staining, Regaud-fixed tissues stain poorly with carmine, alcohol fixation causes weak staining with formulas employing stains other than carmine, and osmic acid prevents carmine staining.

It is well to keep stock solutions of various fixatives on hand since most of the fixing solutions are stable.

Zenker fluid:

Stock Zenker solution:

Mercuric chloride	5 gm.
Potassium dichromate	2.5 gm.
Sodium sulfate	1 gm.
Distilled water	100 ml.

Dissolve by heat.

Zenker solution for use:

Stock Zenker solution	100 ml.
Glacial acetic acid	5 ml.

Add the acetic acid when ready for use and mix.

1. Fix tissues for 12-16 hr.; then wash overnight under a gentle stream of tap water to remove all the excess potassium dichromate.
2. Transfer to 70% alcohol and allow tissues to remain in 70% alcohol until ready to be dehydrated, cleared, and embedded.
3. Simple washing is not always sufficient to remove the mercury. **To remove the mercury,** place the tissue in 70% alcohol with enough iodine to give it a good port-wine color, and leave it in this solution until all the iodine color disappears. Renew the iodine after it disappears and leave the tissue in the solution. Continue renewal of the iodine until the tissue no longer decolorizes it, at which time all the mercury has been removed from the tissue.
4. Then place in 70 or 80% alcohol until all the iodine color leaves the tissue, and proceed from this step in the usual manner for dehydration, clarification, and embedding.

Helly fluid: Helly fluid is a slight modification of Zenker fluid. Helly omitted the glacial acetic acid and replaced it with 10% formalin, which is added to the mixture at the time of use.

Mercuric chloride	5 gm.
Potassium dichromate	2.5 gm.
Sodium sulfate	1 gm.
Distilled water	100 ml.
Formaldehyde, 40% (formalin)	10 ml.

1. Fix tissues for 8-16 hr. Wash for 12-16 hr. under a gentle stream of water.
2. Transfer to 70% alcohol. Allow to remain until ready to embed.

Regaud formol dichromate fluid: Regaud solution gives excellent results with mammalian tissues. It costs less and penetrates better than chrome-osmium fixatives. It is unstable and must be prepared fresh at the time of use.

Potassium dichromate, 3%	80 ml.
Formalin	20 ml.

1. Fix tissues for 4 days, changing the fluid every day.
2. Wash in running water for 16-24 hr.
3. Transfer to 70% alcohol.

Formaldehyde (formalin): Formaldehyde is a gas, soluble in water to the extent of 40% by weight. It has been in general use for many years as a fixing reagent. It penetrates and fixes the tissues quickly, thereby enabling one to cut and stain satisfactory frozen sections. Formaldehyde preserves fat, myelin, nerve fibers, amyloid, hemosiderin, and various organisms. The best strength is a 10% solution of formalin prepared by adding 1 vol. commercial 40% formaldehyde (formalin) to 9 vol. tap water or saline. To prevent development of formic acid, which is injurious to the tissue, add 5-10 gm. calcium carbonate, lead oxide, or bicarbonate of soda to 1 gallon stock formalin. When specimens are to be kept for long periods, it is essential to place a few marble chips in the container.

Formaldehyde vapor is injurious to the eyes, nasal mucosa, hands, and other exposed parts of the body, and contact should be avoided as much as possible. Allergic persons may develop formaldehyde dermatitis, which may become very serious. The lesions persist for months.

The following solution will **relieve the lesion caused by formaldehyde:**

Urea crystals, c.p.	5 gm.
Ammonium phosphate, c.p.	1 gm.
Distilled water	100 ml.

For protozoologic work, fixation in Bouin or Schaudinn solution is preferable.

Picric acid: Picric acid coagulates protoplasm. It is used as a saturated aqueous solution. Solubility is between 0.5 and 1% at room temperature.

Bouin fluid:

Picric acid, saturated aqueous solution	75 ml.
Formaldehyde, 40%	25 ml.
Glacial acetic acid	5 ml.

This solution keeps well. It has considerable penetrating power and causes very little shrinkage. Picric acid, which stains the tissue yellow, is extracted in the dehydration process. It is not a good fixative for cytologic work or for inclusions such as Golgi bodies and mitochondria because it distorts or dissolves them. It is a good fixative for small skin lesions.

Schaudinn solution:

Mercuric chloride, saturated aqueous solution	2 parts
Absolute or 95% alcohol	1 part

Glacial acetic acid in proportion of 10%.

Alcohol: Alcohol hardens and dehydrates tissue at the same time, preserves bacteria, fibrin, pigments, and elastic fibers, and is the only reagent (absolute alcohol) to preserve glycogen.

Tissues to be kept for a considerable period in alcohol should be placed in dilute (70%) alcohol. If left longer than 48 hr. in absolute alcohol, they become so hard that section cutting is almost impossible.

The material fixed in alcohol should not be over 0.5 cm. thick. Strong alcohols, 95% or absolute, harden only the surface of the tissue, so that deeper parts are not penetrated. To avoid this a 70% alcohol, gradually increased to 95%, should be used.

Alcohol-formalin mixture:

Alcohol, 95%	100 ml.
Formalin	50 ml.

Isopropanol: Anhydrous isopropanol has been successfully substituted for absolute ethyl alcohol as a dehydrator in the preparation of paraffin blocks and staining procedures. It is recommended for its economy. It may be obtained through any laboratory supply house.* It must be understood, however, that it cannot be used successfully in the preparation of alcoholic solutions, such as acid alcohol, ether-alcohol mixture for dissolving celloidin, or alcoholic dye solutions.

Bichloride of mercury and formaldehyde combination: A saturated solution of bichloride of mercury, when used alone, causes serious shrinkage of the cells. A combination with formaldehyde has been found to be a very useful fixing reagent.

Bichloride of mercury, saturated aqueous solution (8 gm. to 100 ml. water)	90 ml.
Formaldehyde, 40% commercial	10 ml.

1. Fix thin pieces of tissue from 6-12 hr.
2. Place in 70% alcohol, dehydrate, and embed in the usual manner.

Osmic acid solutions: Osmic acid is an expensive reagent. It is sold in sealed glass tubes of 0.5 and 1 gm. The solution is prepared by dissolving 1 gm. osmic acid crystals in 100 ml. pure, dust-free distilled water. The solution of osmic acid is easily spoiled by formation of black precipitates. This can be partially remedied by the addition of a few drops of 10% chromic acid or 10 vol. hydrogen peroxide. The vapor of osmic acid is irritating to the eyes.

Osmic acid is used to demonstrate fat, but its penetrating power is poor. For this reason Flemming and Marchi have combined osmic acid with other reagents, such as chromic acid and potassium dichromate, to increase its penetrating power.

Flemming solution:

Osmic acid, 2% aqueous solution	20 ml.
Chromic acid, 1% aqueous solution	75 ml.
Glacial acetic acid	5 ml.

1. Fix thin blocks of tissue, not over 2 mm. thick, for 12-24 hr.
2. Wash under a gentle stream of water overnight, place in 70% alcohol, dehydrate, and embed in the usual manner.

The stock solution keeps poorly; therefore, it is best to keep the osmic and chromic acid solutions in separate bottles and mix them at the time of use. The amount of solution should be about 5 times the volume of tissue.

Marchi fluid:

Potassium dichromate	2.5 gm.
Sodium sulfate	1 gm.
Water	100 ml.

1. Mix 2 parts of this solution with 1 part 1% aqueous osmic acid.
2. Fix thin blocks of tissue, not over 2 mm. thick, 5-8 days. Wash under running water overnight, place in 70% alcohol, dehydrate, and embed in the usual manner.

The stock solution is not stable; therefore, these solutions should be mixed at the time of use.

Potassium dichromate: Potassium dichromate forms insoluble compounds with cell proteids and lipoidal bodies. A 3% solution in distilled water is employed. Saturation point is about 12 gm. in 100 ml. distilled water.

Xanthydrol reagent: Acetic acid combined with xanthydrol is used to preserve and demonstrate urea crystals in tissue and smears.

Xanthydrol crystals	5 gm.
Glacial acetic acid	100 ml.

1. Fix blocks of tissue, not over 2 mm. thick, for 6 hr. cold or 1½ hr. at 70° C.
2. Wash under running water for 5 min. and place in 10% cold formalin for 12 hr. or ½ hr. at 70° C.

Acetone: Acetone as a fixative has limited application. It is used as a rapid fixing solution of brain tissue for the diagnosis of rabies and as a fixative for enzymes.

Susa fixative:

Mercuric chloride	4.5 gm.
Sodium chloride	0.5 gm.
Trichloracetic acid	2 gm.
Glacial acetic acid	4 ml.
Formalin	20 ml.
Distilled water	80 ml.

1. Place the tissue in Susa fixative 5-48 hr., depending on the size.
2. Wash in tap water 3-5 hr., changing the water frequently. Place in 50% alcohol for half a day, changing the alcohol several times.
3. Transfer to 70% alcohol for half a day, changing several times.
4. Transfer to 95% alcohol for half a day, changing alcohol several times.
5. Place in absolute alcohol not longer than 1 hr.
6. Clarify in pure xylol. The tissue must become transparent.
7. Embed in the usual manner.

This fixative is used for sections to be stained by Giemsa stain (Schilling method, modified by Gradwohl).

Acetic acid fixative:

Solution A:

Acetic acid	1 part
Absolute alcohol	3 parts

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*Union Carbide & Carbon Co. product has been found superior.

Solution B:

Acetic acid	1 part
Absolute alcohol	6 parts
Chloroform	3 parts

Solution B is good for lymph and glandular tissues. The chloroform helps penetration, and the tissue is fixed more rapidly. When using alcohol-containing fixatives, omit all steps preceding the solution of the strength of alcohol used.

Wash out the acid alcohol with 95% alcohol.

If tissue is submitted in alcohol, put it in 95% alcohol or in the acid alcohol solution above, since the exact strength of the alcohol fixative must be known.

Mueller solution:

Potassium dichromate	2.5 gm.
Sodium sulfate	1 gm.
Water	100 ml.

Orth mixture:

Formalin	1 part
Mueller solution (above)	9 parts

DEFORMALINIZATION

To remove formalin from formol-fixed sections and tissues: At times it becomes necessary to refix formol-fixed tissues or sections in some other fixative, such as Zenker fluid, for special stains. The following technic has given satisfactory results in the majority of cases.

For sections:

1. Deformalinize for 1 hr. in ammonia water, 40 drops of strong ammonium hydroxide to 100 ml. water.
2. Wash 1 hr. in running water.
3. Fix 1 hr. in Zenker or Helly fluid.
4. Wash 1 hr. in running water.
5. Stain with desired method.

For blocks:

1. Deformalinize for 2 days in an oven at 40° C. in ammonia water, 40 drops ammonium hydroxide to 100 ml. water.
2. Wash for 24 hr. in running water.
3. Fix in Zenker fluid for 12 hr. or in Helly fluid for 5 hr.
4. Wash for 12 hr. in running water.
5. Place in 70% alcohol until ready to embed.

DECALCIFICATION

Bone tissues to be decalcified should be sawed with a fine saw into thin slices so that they will decalcify quickly. Very dense and hard bones should not be more than 2 or 3 mm. thick; softer tissues need not be less than 4-6 mm. thick.

1. Fix bones and calcareous tissues thoroughly in alcohol-formalin mixture, 10 parts formalin in 90 parts 95% alcohol, or in Zenker fluid. Leave in alcohol-formalin solution 2-4 days or in Zenker fluid 16-24 hr. before submitting to the decalcifying process.
2. After decalcification, remove the acid with with a weak solution of lithium carbonate, sodium sulfate, or any suitable neutralizing reagent and place in 70% alcohol or isopropanol to prevent further swelling.
3. For the preparation of frozen sections, remove from 70% alcohol, and fix in 10% formalin for 24 hr.

Formic, nitric, hydrochloric, acetic, picric, sulfurous, trichloracetic, and sulfosalicylic acids and others are used alone or in combination with other chemicals for decalcification purposes.

Of all the reagents, formic, trichloracetic, and nitric acids are the most useful and important. Formic acid combined with trichloracetic acid acts quickly without swelling the tissues and does not interfere with any of the staining processes. The nuclei of the cells stain beautifully with the hematoxylin-eosin method. Nitric acid also acts quickly and without much swelling but affects the staining property of the cells, and often the nuclei fail to retain the blue hematoxylin stain.

Decalcification of bone for paraffin sections

1. Place a specimen in a large volume of 5% nitric acid and change daily until the specimen becomes soft. This requires up to 5 days. Be sure to use a very large quantity of solution.
2. Wash in running water 24 hr.; then dehydrate in alcohols from 80% to 95% to absolute.
3. Place in cedarwood oil for clearing until the specimen becomes transparent and sinks.
4. Place in xylol for 30 min.
5. Place in paraffin in oven for 2 hr.
6. Embed in paraffin and cut sections as usual.
7. Stain preferably with eosin-methylene blue.

Instead of using 5% nitric acid and washing in running water for 24 hr., the following decalcifying solution may be used, taking care to use 100 times the volume of tissue:

Hydrochloric acid	4 ml.
Glacial acetic acid	3 ml.
Chloroform	10 ml.
Water	10 ml.
Alcohol, 95%	73 ml.

Pass tissue immediately into fresh absolute alcohol (2 changes) and then into cedarwood oil; the rest of the technic is as above.

Rapid method of decalcification:

Formalin	1 part
Nitric acid	1 part
Water	9 parts

1. Place in this solution for fixation.
2. Wash in running water and then begin dehydration in 80% alcohol and continue through the alcohols a little more slowly.

Bone tissue or calcified nodules

Decalcify in 5% nitric acid or in the following solution:

Formaldehyde, 40%	10 ml.
Distilled water	90 ml.
Nitric acid	20 ml.

If the bone is not too large, keep in the solution for 2-3 hr. It may be kept in the solution as long as 48 hr. without harm. There should be at least

10 times as much solution as tissue. Change the solution frequently.

1. Wash in running water 12-24 hr.
2. Place in 80% alcohol.
3. Proceed as for paraffin embedding.
4. If tissue is soft, it is ready for processing.

Decalcification of temporal bones—Krajian

This method employs a controlled shaking machine and a modified decalcifying fluid. It is applicable to smaller bones.

Decalcifying solution:

Formic acid, 88%	500 ml.
Nitric acid, concentrated	100 ml.
Tergitol (sodium heptadecyl sulfate)	2.5 ml.

1. Dilute the formic acid with 1000 ml. tap water.
2. Add the Tergitol and mix well.
3. Dilute the nitric acid with 900 ml. tap water.
4. Mix thoroughly with the dilute formic acid.

Warning: Do not mix formic and nitric acids in concentrated form, otherwise an explosion will occur.

Decalcification method:

1. Place temporal bones in a 200 ml.–capacity specimen bottle, and fill the bottle with the decalcifying solution, pouring the solution slowly. As the fluid comes into contact with the bone, immense amounts of bubbles arise. Wait for the bubbles to disappear.
2. Stopper the bottle tightly and place on a shaking machine at 35 oscillations. The shaking machine is the standard Kahn shaker controlled with Variac.*
3. Change the solution twice at 8 hr. intervals.
4. Prick the specimen superficially with a needle once a day.

It requires 8-12 changes for a temporal bone to be completely decalcified depending on the amount of calcium present and hardening of tissue due to other causes.

This method is also applicable to smaller bones removed at surgery or autopsy and requires only 1 or 2 changes for complete decalcification.

5. After complete decalcification neutralize the specimen by placing it in 1% lithium carbonate for 24 hr.
6. Wash for 16-24 hr. under gently running tap water.
7. Place in 40% ethyl alcohol until ready for dehydration and embedding.

This is the most efficient fluid ever devised for decalcifying. If a shaking machine is not available, the fluid may be used by changing the solution twice a day until the bone tissue is completely decalcified. Refer to p. 1753.

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*General Radio Co., Cambridge, Mass.

Chapter 85

Equipment for cutting sections

ARAM A. KRAJIAN

MICROTOMES

The instrument used for cutting thin sections of tissue or other material for preparation for microscopic examination is called a microtome. These instruments are designed to cut material extremely thin, from 1 to 60μ, and may or may not be provided with mechanical feeding mechanisms. There are various types of microtomes. The **rotary type** is used for cutting paraffin-embedded sections. The **sliding microtome** is used for frozen sections and for special purposes, principally for cutting celloidin-embedded and large, refractory paraffin material. In this microtome the knife moves against the object, a slow but steady motion being required to manipulate the instrument.

The **freezing microtome** is used to cut unembedded, formalin-fixed tissues, which are frozen to the proper consistency with carbon dioxide. This microtome is operated by rotation of the flywheel, causing a reciprocal motion of the knife over the block, the thickness of the section being automatically regulated by a ratchet-feed wheel. See Figs. 520 and 524.

The Minot **simplified rotary microtome** was designed in accordance with the wishes of the late Dr. Charles S. Minot of Harvard University, to provide an instrument with the efficiency of the more elaborate rotary microtomes but of simpler construction. The rotation of the large wheel causes the block holder to move vertically, up and down, through a distance of about 3 in. The object holder consists of a disk 25 mm. in diameter which can be oriented to any angle and which moves on a vertical slide actuated by a crank that is operated by the drive wheel. The knife is held in a fixed position at the base. This position is adjustable to and from the object and from side to side to permit the use of nearly the entire cutting edge. The feeding apparatus operates automatically and may be adjusted to cut any thickness from 2 to 26μ, at intervals of 2μ.

There is a specially designed microtome for frozen sections. It is known as an **automatic microtome** with a swinging carriage. The peculiar motion of the knife makes this microtome especially desirable for cutting celloidin sections, when equipped with freezing attachment. It is not adapted for paraffin work. The extreme ends of the knife rest in the carrier, and as the lever handle moves the swinging arms, the knife describes a flattened curve, corresponding to the double movement of freehand sectioning. The knife used with this microtome should be 110 mm. long. The automatic feed mechanism may be used or, by means of a lever extending to the graduated scale on the front of the main frame base, it may be thrown out of action. Better results are obtained in frozen sections if the automatic feeding mechanism is detached and the microtome is operated by hand.

Complete diagrammatic sketches are supplied with each microtome, giving directions for use.

MICROTOME KNIVES

There are 3 types of microtome knives: (1) the plane-wedge knife used for cutting frozen sections, (2) the biconcave type, used for paraffin work with the rotary microtome, and

Text continued on p. 1650.

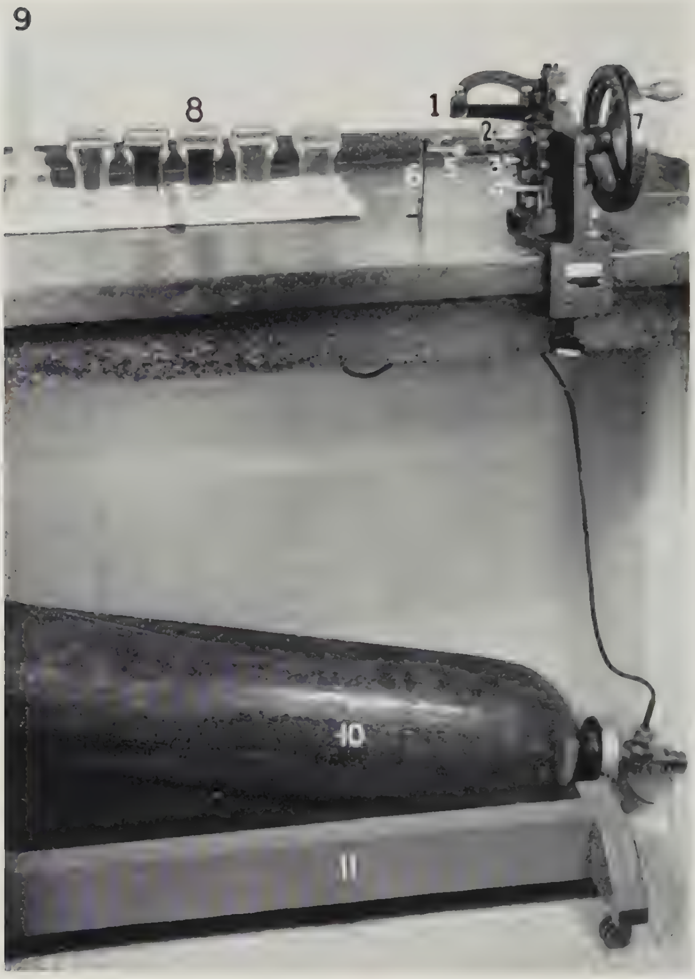

Fig. 520. Freezing microtome. **1,** Knife mounted on a sliding arm and tightened with 2 screws at an angle of about 45°; **2,** block holder directly under the knife; **3,** screw to tighten the block holder; **4,** ratchet feed wheel; **5,** small valve to admit CO_2 into chamber of block holder; **6,** copper feed pipe connecting CO_2 gas to microtome; **7,** flywheel; **8,** staining dish; **9,** large Pyrex test tube for boiling formalin; **10,** cylinder containing 50 lbs. liquid CO_2; a tank using solid CO_2 (dry ice) is also available; **11,** wooden stand for cylinder. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

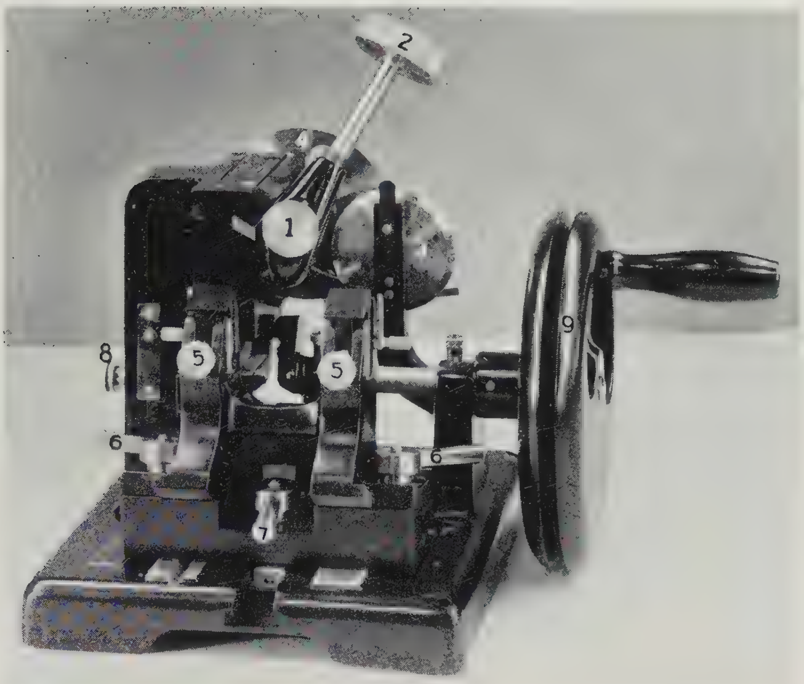

Fig. 521. Paraffin microtome (small type). **1,** Metal block holder; **2,** block holder locking screw; **3,** micrometer adjustment for thickness of section; **4,** ratchet feed wheel; **5,** knife-holder screws; **6,** clamp nuts for knife stand regulator; **7,** knife stand clamp nut; **8,** pillar locking pin; **9,** flywheel. This microtome is operated in same manner as the freezing microtome. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

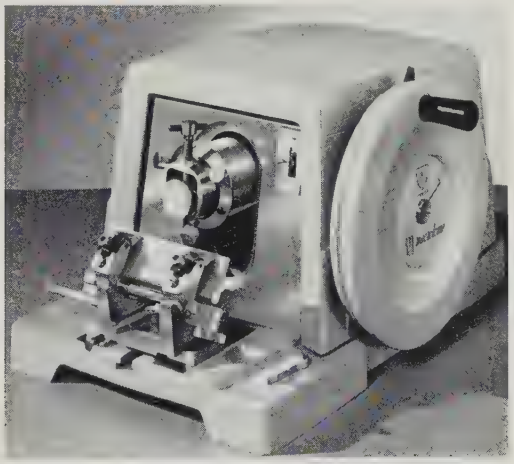

Fig. 522. AO Spencer precision rotary microtome. (Courtesy Arthur H. Thomas Co., Philadelphia, Pa.)

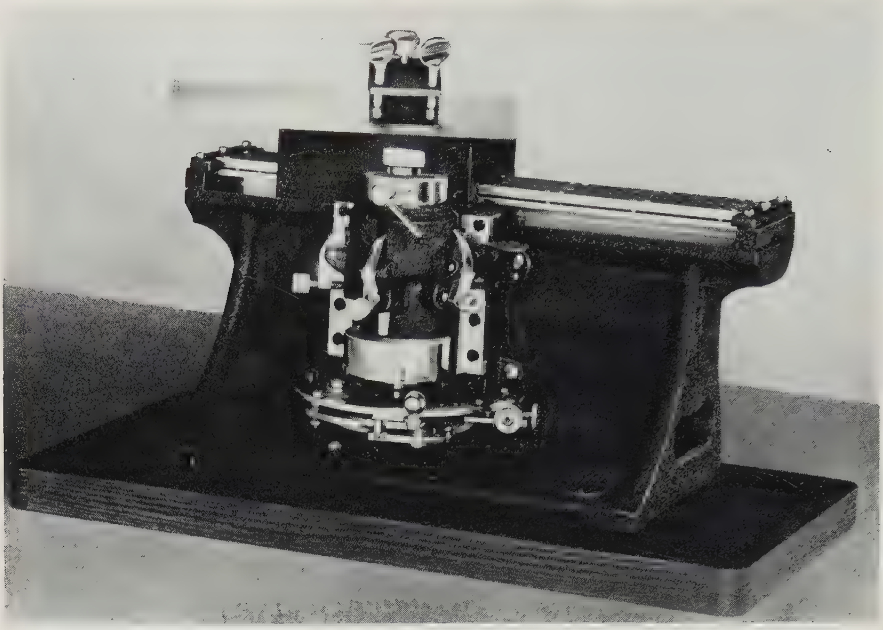

Fig. 523. AO Spencer sliding microtome. (Courtesy Arthur H. Thomas Co., Philadelphia, Pa.)

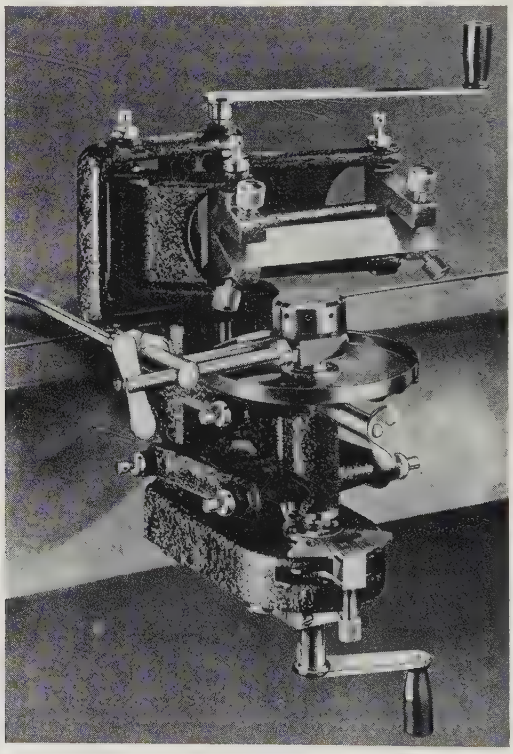

Fig. 524. AO freezing microtome. (Courtesy American Optical Co., Instrument Division, Buffalo, N. Y.)

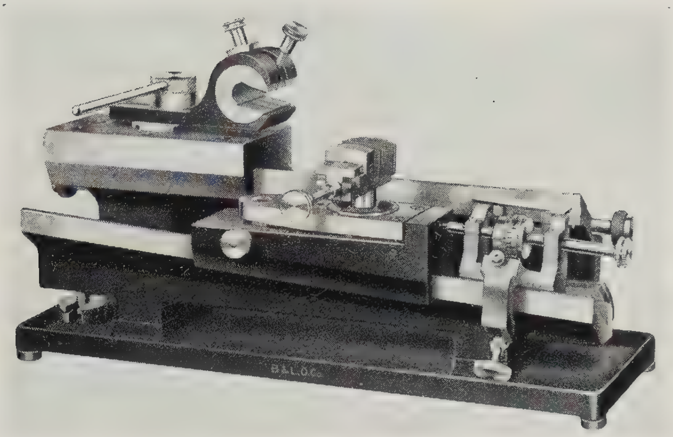

Fig. 525. Celloidin microtome showing knife, knife-holder screws to regulate the angle of the knife, screw to regulate the block holder, screw to regulate the angle of the block holder, micrometer adjustment for thickness of the section, ratchet feed wheel, and fly-wheel. This microtome is operated in the same manner as the paraffin microtomes. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

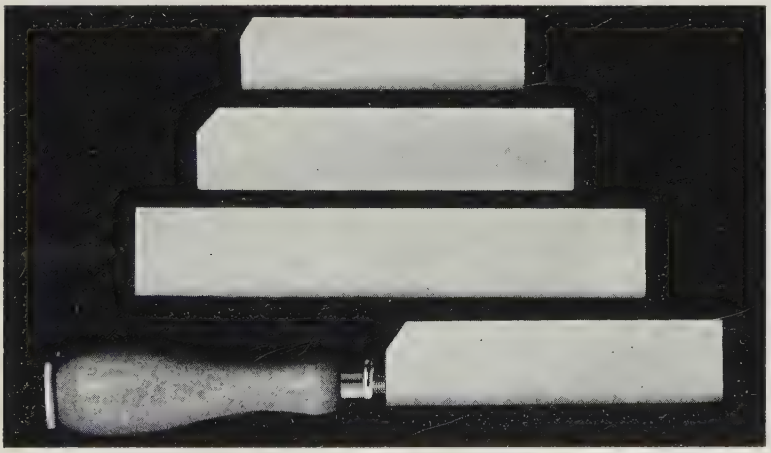

Fig. 526. Microtome knives, various sizes, one with handle attached. (Courtesy Bausch & Lomb, Inc., Rochester, N. Y.; from Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

(3) the plane-concave type, used for celloidin work with the sliding microtome. Standard size knives are supplied for freezing and celloidin microtomes. For the paraffin microtome it is better to have a larger knife, for after one position of the edge becomes dull it will be necessary to move along the knife and bring a sharper edge into action. This will save the time and trouble of frequent honing and stropping. Razor blades in special holders have been found unsatisfactory.

To sharpen a microtome blade*†: Prepare the microtome knife for honing by screwing the handle into the threads provided at one end of the knife; then slide the sharpening back onto the knife. This sharpening back raises the knife and establishes the correct angle of the cutting facets. Take care to put the proper back onto the knife. Each knife has its own sharpening back; these backs should not be interchanged between knives.

Look at the cutting edge of the blade under the low-power objective of the microscope to examine for nicks. If the edge is appreciably nicked or not straight and even, it may be straightened by rubbing gently on a smooth stone, holding the knife at right angles to the stone. This will establish a straight flat surface along the cutting edge. Now hold the knife so that a strong nearby light reflects from the cutting edge. There will be a broad line of reflected light. Honing will remove small amounts of the metal first from one side of the edge and then from the other until nothing is left but the sharp cutting edge.

Use a fine hone or a piece of plate glass with appropriate abrasive. Lubricate the hone with a solution of neutral soap. Pass the knife over the stone, the cutting edge forward. In other words, proceed forward with the cutting edge of the blade, turn the knife over **across its back,** not over its edge, and bring it back, maintaining sufficient pressure to hold the edge evenly against the hone. If no pressure is used, the hone does not take hold well. If too much pressure is used, the edge is rounded. Gauge the amount of pressure in accordance with the hardness of the knife and the fineness of the hone. By watching the reflection of light from the edge of the knife, the progress of honing may be ascertained. When the reflection is very slight, examine the knife under the microscope to make sure that the edge is smooth and well sharpened.

The glass plate should be at least ⅜ in. thick or preferably thicker. Use plate glass 12-14 in. long and at least 1 in. wider than the length of the knife. The **abrasive** may be purchased graded to size, or it may be prepared by making a suspension in water and setting it in a tall cylinder. The heavy particles fall rapidly to the bottom; the lighter particles fall more slowly. The time of standing must be gauged by experience. Use the middle portion containing particles of proper size. For grinding, use an average size of 20μ, with none larger than 40μ. For final polishing of the facet, particles averaging 4μ and less than 8μ may be used. Suspend the particles in a solution of neutral soap (about 1%). Gradation of abrasives is accomplished by pouring the supernatant liquid of the abrasive preparation serially from one container to another. The final supernatant liquid contains the finest particles.

To smooth the edge when nicks must be removed, No. 303 or 304 corundum may be used. Levigated alumina or No. 305 corundum is suitable for ordinary sharpening and after the above grinding compounds. Diamantine or similar material should be used for the final polishing of the cutting facets.*

To polish a microtome blade: Prepare the knife as for honing. Use the finer abrasive. Very little is required. Proportions are soon learned by experience. Avoid too much abrasive. Wash the plate free from abrasive occasionally to rid it of large particles and any bits of steel freed from the edge of the blade. Hold the edge evenly against the glass; do not force against it, or the edge will become round rather than sharp. If the facets are irregular, the grinding or polishing is uneven. Use fresh abrasives from time to time during the polishing operation. Toward the end use more dilute abrasives than at the beginning. Examine under the microscope. If the edge shows that the knife is well honed, stropping is unnecessary.

To strop a microtome knife: Do not strop too much or the edge may become curved. Strop the knife from heel to toe. Clean the strop and free it from any abrasive larger than about 2μ. The strop should be mounted on a board or held against some other surface so that it will not give as the knife is pulled over it. If the strop gives or if the knife is pressed against the strop, the edge will be rounded and spoiled. Hold the knife firmly against the strop, with light pressure. After a few strokes, first on one side and then on the other, examine the edge under the microscope. Discontinue stropping just as soon as an edge appears smooth. It is unnecessary to test the knife with the fingernail, hairs, or other material; this only tends to dull the knife. The test of the knife is whether or not it cuts effectively.

If a knife needs touching up but does not need a thorough honing, use a strop with one side embedded with diamond dust or other coarse abrasives. Then strop on the smooth side.

After the knife has been used, it must be cleaned and wiped dry; then put away in its proper place. If the atmosphere is corrosive, it may be necessary to oil the knife to prevent corrosion. Use only a good grade of light neutral oil.

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*The Micro Sharp Co., Niagara Falls, N. Y. and Toronto, Ontario, Canada, does excellent work in sharpening microtome blades. The author recommends this company to those who do not sharpen their own blades.

†For much of the material on the care and use of microtome we are indebted to Dr. O. W. Richards, Research Biologist, of American Optical Co., Scientific Instrument Division; this material is contained in a pamphlet by Dr. Richards, "The effective use and proper care of the microtome," published by Spencer Lens Co., Buffalo, N. Y.

*Dr. A. Klein, Norton Co., has suggested that a good polishing compound similar to Diamantine may be made by calcining pure ammonia alum in an **unglazed** clay or fused silica crucible for 30 min. at 2300° F. The crucible should be only ¼ full as the alum swells during heating. The yield is about ⅛ of the original weight.

If the knife is in such poor condition that it must be ground, it must be sent back to the manufacturing house that supplied it*; grinding should be done only by experts.

Krajian's method of sharpening a microtome blade: If the knife is dull but free from nicks, it may be sharpened by stropping. Frequent stropping of the blade usually keeps it in good condition. If, however, the knife is used for a great many blocks, stropping alone will not keep it sharp enough, and one must resort to honing. If the knife must be ground, it should be sent back to the supply house where it was purchased*; grinding should be done only by experts.

For honing use a No. 108 carborundum stone. Before honing, cover the surface of the stone with fine machine oil. Be sure that the back is in place on the knife and that the handle is firmly screwed into the blade. Place the knife at one end of the hone; then without any pressure except that caused by the actual weight of the knife gently push it to the other end so that the sharp edge is proceeding forward. Turn the knife over (against the back, not across the cutting edge) and push it back on its other side to the other end of the hone. Continue until the nicks are gone or until it feels sharp to the thumb. This honing can be accomplished in about 5-10 min. if properly carried out. If there are deep nicks in the knife, then exert firm pressure while honing.

Use a strop with both rough and smooth sides. When stropping, turn the knife over at the end of every stroke so that both sides are stropped in turn. Usually it requires about 1 dozen strokes on the rough side and about 2 dozen strokes on the smooth surface to obtain the proper cutting edge. Strokes should be gentle. Do not exert pressure. The weight of the blade is sufficient when stropping.

GENERAL FACTS ABOUT CARE OF MICROTOMES

Microtomes should be kept free from dust. Always cover when not in use.

Be sure that all sliding parts are clean. Oil these parts occasionally with a good grade light machine oil. Be careful not to over-oil or to add the lubrication to dirty mechanisms. Place oil on the main bearing block through the oil inlet provided. Occasionally oil the feed screw.

Like all other precision instruments, the microtome requires gentle handling. Do not put undue pressure on the flywheel or other portions of the apparatus which are handled frequently. If the alignment of the microtome is interfered with by constant pressure at certain points, the instrument will have to be sent back to the manufacturer for repair. To tighten connections use a smooth wrench, not a pair of pliers. We have seen instruments literally "chewed up" with the erroneous use of pliers on the soft metal.

After considerable use[2] the microtome may cut sections irregularly because of wear. Turn the handle of the feed mechanism. A distinct drag or tension should be felt. When it turns freely, it should be tightened. This usually stops the irregularity of the cutting. Tighten the friction screws on the friction bracket one full turn at a time until the friction is regained. If the feed pawl drags over the teeth of the ratchet wheel, adjust the friction block in the pawl lever by turning the 2 screws to be found back of the ratchet wheel. Turn these screws one at a time until the pawl lifts out, clear of the teeth of the ratchet wheel on the down stroke.

Do not disturb the feed screw mechanism. Exercise great care in returning the feed screw to its initial position after it has reached the end of its excursion and stopped advancing the specimen. Avoid use of force when handling the feed screw.

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*See footnote on p. 1650.

Chapter 86

Special technics and specimen mounting

ARAM A. KRAJIAN

METHODS OF AFFIXING SECTIONS TO SLIDE

Mayer albumin fixative

This method was first described by Von Henniguy. It was later modified by P. Mayer and still later by Von Reinke.

Procedure:

1. Beat the white of an egg until it is stiff and dry. Let stand until clear.
2. Filter through filter paper.
3. Add an equal volume of glycerin.
4. A crystal of thymol may be added as a preservative if desired.

To use the fixative:

1. Rub a drop of the fixative briskly over the surface of a slide but do not allow to dry.
2. Tease the section onto the slide in a drop of water. Remove the excess water by gentle blotting and place the section at 37° C. in an incubator overnight or gently warm in a flame, or let the section stand at room temperature overnight.

Preferred method:

1. Use equal parts of egg white and glycerin.
2. Beat thoroughly with a glass rod and filter through several thicknesses of gauze.
3. Add a crystal of thymol as a preservative.

Alternate method:

1. Mix equal parts of blood serum and glycerin with 10% formalin. To use, place a faint trace of the mixture on a slide.
2. Warm gently in a Bunsen burner, keeping the heat at about 70° C.
3. Pour distilled water over the slide.
4. Place the section in the distilled water and straighten it.
5. Remove the excess water with filter paper.
6. Place the slide in an incubator at 37° C. for 3-4 hr. or have the section in slightly warm distilled water.
7. Float a cover glass on the section to pick it up.
8. Heat the preparation to 70° C. and continue as described below.

Schneidau method[3]

Schneidau method 1

Albumin solution—Schneidau:

Egg white	from	1 egg
Glycerin		50 ml.
Sodium salicylate		1 gm.
Distilled water		1500 ml.

Mix thoroughly and filter.

1. Place the solution in a narrow-mouthed jar equipped with a 2-hole rubber stopper. Through 1 hole fit a bent glass siphon to which has been attached a rubber hose containing a small pipet at the end, and a spring clamp. Place the apparatus on a shelf several feet higher than the laboratory table, start the siphon, and then shut off the flow with the spring clamp.
2. To use, assemble slides, squeeze the clamp, and allow just enough fluid to run onto each slide to float the section. Float sections by means of a section lifter, spread out by heating, and blot up excess fluid. Set aside to dry.

With this method the danger of using too much or too little fixative is practically eliminated. The sections do not entirely dry because of the glycerin and may be left on the slides for months before mounting without danger of drying or cracking.

Schneidau method 2

1. Float the sections onto the surface of slightly warmed water to spread them and then place them on the slides and dry without using any albumin.
2. Use a pan of approximately 15 × 10 × 3 in. Fill it with water and heat to about 40° C. While the water is heating, cut the ribbon of sectioned tissue into squares of one section each.
3. Take the sections up one at a time on a section lifter and drop them onto the surface of the water. Take care that one section does not fall on top of another, since they cannot be separated once they are in the water. No more than 100 sections should be worked at one time in a pan of the dimensions listed.

4. Dip a slide into the water at an angle of about 50° until the water level is about 0.5 cm. above the midline of the slide.
5. Take a straight probe or needle, and, catching any of the sections by one edge, pull it over and hold it against the slide with the needle and pull both slide and needle out of the water together. The water will run out from under the section and leave it in about the center of the slide, depending, of course, upon how close to the center the section was held with the needle.
6. Repeat until all of the sections have been placed on slides; then place the slides in a drying oven overnight. If no drying oven or incubator is available, then let the slides stand for 3 days to ensure complete evaporation of the water.

Contrary to the belief of many, the sections will not wash off the slides provided they have completely dried and do not receive rough handling in the staining and mounting processes. One precaution to be observed is to see that there are no bubbles on the surface of the water that is used, since a bubble under a section will cause it to crack when dry or to wash off of the slide.

This method is essentially a time-saving method but is not as reliable as the first method described. However, if done carefully, it should not cause any trouble.

SPECIALIZED PREPARATION OF TISSUE

Technic of making large sections of organs as originated by Christeller

A specially constructed sliding microtome* with a large freezing table is used.

Slides are photographic plates 9 × 12 cm. Cover glasses slightly smaller* are used. These are approximately 8½ × 11 cm. Staining dishes are rectangular-shaped museum jars. There should be about 12 of these. Tissues should be hardened in 10% formalin.

Procedure:

1. Place a moistened piece of good grade lens paper on the microtome block.
2. Place the tissue to be cut on this paper and press down.
3. Cover with the freezing hood and freeze slowly.
4. When the tissue is frozen, remove hood and cut.
5. Moisten the fingers. Cut one slice at a time, lifting the slice with the fingers as you cut. Be careful not to cut your fingers during this process.
6. After removing from blade place the sections in water, using your fingers.
7. Place sections on plates with bent glass rod with blunt end. This is not easy. It takes several days' practice to become efficient in this process. There must not be a single bent place on the section.
8. Dehydrate with absolute alcohol. Use a large quantity of alcohol.
9. Press out all air bubbles with rod.
10. Dehydrate 3 or 4 times.
11. Cover with a thin layer of very thin celloidin. (Make this celloidin from stock solution, using 2 ml. to 100 ml. of equal parts of alcohol and ether.) Let it be thin enough when placed on the back of the hand to make an extremely thin coating. Run excess back into bottle.
12. Wait until it is almost air dried but **not** dried.
13. Place solutions for staining in museum jars (11 × 15 cm.).
14. Lay the specimens in water directly.
15. Place in Boehmer hematoxylin for 3-4 min.
16. Then place in distilled water.
17. Place in hydrochloric acid-alcohol, 1 ml. hydrochloric acid to 100 ml. 70% alcohol.
18. Place again in distilled water. Repeat in fresh water to remove acid. Section becomes blue.
19. Stain in aqueous eosin solution.
20. Wash in distilled water; then carry through:
21. Alcohol 70%, 1 min.
22. Alcohol 95%, 1 min. Do not use absolute alcohol, since this redissolves the celloidin.
23. Terpene oil (Kahlbaum) or carbolxylol, 1 min.
24. Xylol.
25. Canada balsam. Put balsam in middle of plate and slightly bend the cover glass to the middle; let it down on both sides and press and put weight on it overnight.
26. Label.

Preparation of small pieces of skin tissue[4]

1. Fix tissues in Bouin solution (p. 1641) overnight. Twenty-four hr. is the maximum fixing time.
2. 9:00 A.M. Wash in 50% alcohol.
3. 10:00 A.M. Wash in 70% alcohol containing 3 drops of saturated solution of lithium carbonate.
4. 12:00 noon. Put in 70% alcohol. The tissue can be left over the week end in this solution.
5. 3:00 P.M. Place tissues in 80% alcohol.
6. 5:00 P.M. Place tissues in 95% alcohol and leave until the following day.
7. Next day, 9:00 A.M. Place in absolute alcohol.
8. 10:30 A.M. Place in amyl acetate.
9. 4:00 P.M. Place in paraffin in the paraffin oven.
10. 5:00 P.M. Pour off the paraffin and leave the tissues outside of the paraffin oven. Never leave tissues in oven overnight. Never re-use any paraffin.
11. Next day, 9:00 A.M. Put in second paraffin.
12. 12:00 Noon. Put in third paraffin.
13. 2:00 P.M. Embed in paraffin.
14. Cut and stain with routine hematoxylin and eosin stain (pp. 1691 and 1692).

Amyl acetate can be used for clearing formalin-fixed tissues as well as those fixed in Bouin fixative.

If the tissue is fixed in formalin, first wash in water from 9:00 A.M. until 10:30 A.M. Then place in 50% alcohol and then in 70% alcohol until 12:00 noon. Follow the technic for Bouin-fixed tissue from this point.

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*E. Leitz, Inc., New York, N. Y.

This method is especially recommended to make satisfactory sections of very small pieces of skin, biopsy, etc.

Preparation of serial slices of the breast

Method of Ingleby-Holly[5]

In relation to diagnosis of disease of the breast it is of paramount necessity to examine as large portions of this organ as possible in order to come to the proper conclusion concerning the pathologic anatomy. In other words, the organs as a whole must first be studied, and later any special area should be minutely examined.

Procedure: Specimen is brought from the operating room or autopsy table.

1. Suspend specimen in a jar of 10% formalin in order to fix it with the normal contour preserved as nearly as possible. Suspending the specimen can be accomplished by means of a gauze sling.
2. Place the jar containing the breast in the refrigerator room and leave it there for at least 24 hr.
3. Remove from the solution, wrap in a wet cloth, and freeze. Freeze rapidly. Use a commercial ice cream freezer for the purpose or place in a freezing unit.

Ingleby and Holly recommend a slicer* that cuts even slices down to about 0.5 mm. in thickness. With this slicer, freezing of the tissue is not absolutely necessary; however, better results are obtained if the specimen is frozen before slicing, especially if the specimen is of soft or uneven consistency, such as fatty breast where skin is present.

4. Stack sections in order. They may be put back into formaldehyde for further fixation or they may be tagged and numbered at once. Some sections are difficult to handle. Receive these in a bowl of water and float onto numbered pieces of paper, and stack. In this way sections can be kept perfectly flat during further fixation.
5. Fix for a few hours, then wash 1 hr. or more in running water, rinse in distilled water, and place in a single layer in the following filtered solution:

Harris hematoxylin	5 ml.
Distilled water	100 ml.

6. Stain for 24 hr., turning once during this time to ensure even staining.
7. Differentiate in acid alcohol.
8. Wash until blue.
9. Place in

70% alcohol . .	1-2 hr.,
95% alcohol . .	6-8 hr. (2 changes),
absolute alcohol .	2-4 hr. (2 changes),
benzine	2-4 hr. (2 changes).

10. Store in mineral oil until required for use. Then transfer to 2 parts oil of wintergreen and 1 part benzol benzoate for about 1 hr. They are then ready to be examined under the dissecting microscope.

An alternative stain is Masson iron hematoxylin. This gives excellent results where material to be stained is not too dense. It is the method of choice if photography is contemplated.

Sections must be returned to the mineral oil after study. Oil of wintergreen is an excellent clearing agent, but it extracts the hematoxylin and the sections fade rapidly. In some cases sections may be examined in mineral oil without clearing. Occasionally, especially with the ordinary microscope, mineral oil gives better results than the more brilliant oil of wintergreen.

Advantages of method:

1. It is possible to examine grossly every part of the tissue. In this way small lesions are not missed, and if it is not desired to stain whole sections, suitable pieces for paraffin blocks are easily picked out.
2. A 3-dimensional view is obtained and structural relations are beautifully shown; no lesion of any importance can be missed.
3. The method is a rapid one. A whole breast could be ready for examination in 5 days or less.
4. Paraffin sections can be made at any time from any part should higher magnification be desirable.

MOUNTING REAGENTS

For the preparation of permanent mounts, Canada balsam and gum damar are in most general use. The author has been using gum damar for many years as a mounting medium for permanent microscopic tissue sections and has prepared more than 250,000 sections in this time. Gum damar has proved to be superior in every way to Canada balsam. The balsam slides dry slowly, and after years of curing they still have a tendency to stick together when stored in contact. The balsam has turned yellow at the edges and under the cover slips after storage, and at the edges of the sections, in a 2 or 3 mm. zone, there is complete decolorization of the nuclear stain. Slides mounted in gum damar are much cleaner, dry more quickly, do not stick together if allowed to dry for a few months before stacking, do not discolor, and do not fade.

Gum damar: Gum damar may be purchased in the crude state from supply houses.

Dissolve the gum in neutral histologic xylol.* Place the ingredients together in a large stoppered bottle in an incubator and stir with a glass rod several times daily until of a syrupy consistency—about 1 wk.

Strain through 4 layers of clean gauze and store in a dark bottle. If the resultant solution is too thin, leave the unstoppered bottle in an incubator until it evaporates to the right consistency. If it is too thick, add more xylol.

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*American Slicing Machine Co., Chicago, Ill.

*That prepared by Eastman Kodak Co. is especially good.

Canada balsam: Prepare a good grade of Canada balsam like gum damar or purchase from any reliable supply house.

Euparol: Euparol is either colorless or green. Use the green for hematoxylin-eosin stains only. It intensifies the microscopic picture. Very often poorly stained sections mounted in green euparol are intensified after mounting.

To mount, take the tissues directly from 95% alcohol, omitting absolute alcohol and xylol steps.

Chloroform and balsam: Dissolve the balsam in chloroform. Use as a mounting medium for sections stained with osmic acid since other mounting reagents for osmic acid preparations cause the stain to fade. The mixture should be of a heavy syrupy consistency.

Cedar oil: Use cedar oil to mount sections stained by the Giemsa method, and use only thick cedar oil.

Clarite: Clarite is a resin dissolved in toluene or in xylol. It should be purchased from a supply house.

Permount: Permount should be purchased from a supply house. It is a very satisfactory mounting reagent.

Colophonium: Colophonium is sold in solid form. Dissolve in oil of turpentine. It is used for mounting blood films stained with Wright stain. Prepare it in the same way as gum damar.

Glycerin jelly—Kaiser:

Gelatin (French sheet)	10 gm.
Distilled water	60 ml.
Pure glycerin	70 ml.
Liquid carbolic acid	1 ml.

1. Heat the water in a beaker and dissolve the gelatin.
2. Add the glycerin and carbolic acid and stir until smooth.
3. Filter through wet absorbent cotton while hot and store in a cork-stoppered bottle.
4. To melt, place the bottle in a paraffin oven for 15 min.

Gum arabic:

Pure gum arabic	50 gm.
Pure cane sugar	50 gm.
Distilled water	50 ml.
Thymol crystals	0.5 gm.

1. Dissolve the gum arabic and sugar separately and mix together.
2. Add the thymol crystals.
3. Keep in a well-stoppered bottle. It sets quite hard.

Temporary mounting media: For temporary mounts use cedar oil or glycerin and discard the slides after emergency examination.

CLEARING REAGENTS

Clearing reagents are used to make the tissue sections transparent so that they may be seen and differentiated under the microscope. Various reagents of different chemical properties are used. The choice depends mainly on 2 factors, the kind of stain used and the materials in which the tissues have been fixed and embedded.

Most clearing reagents can be used after hematoxylin stain. For celloidin sections, oil origanum and oil of bergamot are recommended. For aniline stains, xylol is the best clearing reagent, but it clears only from absolute alcohol and butyl alcohol.

Carbolxylol: Carbolxylol has a dark straw color. It does not dissolve celloidin and it does not affect hematoxylin. It clears directly from absolute and butyl alcohols, and it will clear from 95% alcohol also if the section is blotted in filter paper before it is placed in carbolxylol. Sections should not be left more than 5 min. in this mixture.

All glassware must be clean and **dry.**

Add 3 parts xylol to 1 part **liquefied** carbolic crystals (phenol) and mix. Do not use liquid carbolic acid since it contains a small amount of water.

Xylol: Xylol is colorless, does not dissolve celloidin, does not affect aniline dyes, clears directly from absolute or butyl alcohols, and will also clear from 95% alcohol if the xylol is used in several applications, the section blotted after each.

Oil origanum: Oil origanum is straw to brown colored and affects aniline stains slowly. It clears celloidin sections very slowly.

Oil of bergamot: Oil of bergamot is light green, clears from 95% alcohol, does not dissolve celloidin, and affects aniline stains slowly, except eosin, which it extracts very quickly.

Oil of cloves: Oil of cloves is straw colored, clears from 95% alcohol, affects aniline stains, especially methylene blue, and dissolves celloidin slowly.

Aniline oil: Aniline oil is colorless when pure and fresh, but it oxidizes rapidly and turns brown. It does not dissolve celloidin, clears from 80% alcohol, and affects aniline stains slowly. A mixture of equal parts of aniline oil and xylol is used to decolorize sections stained by the Gram-Weigert method as well as the Flexner stain for Hansen's bacillus and to clear sections stained for myelin sheaths by the Courville-Krajian method.

Oil of cedarwood: Oil of cedarwood has a pale straw color, clears from 95% alcohol, does not affect aniline stains, and clears celloidin sections very slowly.

Beechwood creosote: Beechwood creosote, formerly much used to clear celloidin sections, is replaced with carbolxylol.

Chloroform: Chloroform has long been used to clear tissues after dehydration. It absorbs a good deal of moisture left in tissues and renders them less brittle.

Benzine: Benzine is too volatile for clearing purposes. Some workers prefer it to chloroform or xylol because it clears quickly, makes the tissue more transparent, and evaporates rapidly from the paraffin bath.

IDENTIFICATION NUMBER UNDER COVER SLIP

A convenient method of labeling slides is shown in Fig. 527. Letter the laboratory specimen number or the name of the patient in

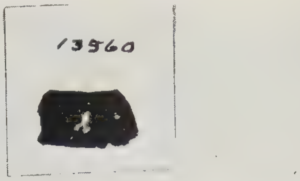

Fig. 527. Identification number under cover slip. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

small letters on very fine tissue paper with India ink (Higgins) and cut out a square. Put a drop of gum damar over the section, place the printed number alongside it, and cover the slide with a cover slip.

PLASTIC MOUNTING OF HISTOLOGIC SECTIONS

Tissue sections may be mounted on 35 mm. film instead of on glass. The sections are then covered with a hardening plastic spray instead of the conventional glass cover slips. The method was devised by Pickett and Sommer.[5a]*

Procedure:

1. Cut film strips of any desired length from the stock roll.
2. Clean by dipping in acid alcohol and dry with a soft cloth. Do not use gauze.
3. Coat the surface of the film with a thin layer of egg albumin while the film is spread out on a clean surface.
4. Cut paraffin sections 5-10μ thick and place a ribbon on the water bath.
5. Maneuver the film under the ribbon and pick up the sections.
6. When the film is covered to capacity, label with a diamond pencil and place in an oven at 56° C. If the films are exposed for a short time to hot water under the film, the sections flatten by themselves.
7. When dry, load the film into 35 mm. film-developing reels.
8. Stain the sections on films in the usual manner. They may also be deparaffinized with xylol, placed in absolute alcohol for 1-2 min., then in 0.5% celloidin 5 min., dried in the air 3-4 min., and placed in 80% alcohol 5 min., then washed and stained as usual.
9. Dehydrate through 95% alcohol and 2 changes of a mixture of ⅓ chloroform and ⅔ absolute alcohol; clear in xylol.
10. Remove one film strip at a time and place face up on a blotter, but do not allow to dry. Be sure the film lies flat to prevent an uneven plastic coat.
11. Apply the plastic spray from one end to the other 2 or 3 times until the surface appears to be smooth. Hold the can containing the plastic approximately 6 in. above the film.
12. Allow the plastic to harden 5-10 min. Repeat 2 or 3 times and leave to dry. A uniform and even spread may be obtained by placing the film strips on the inside of an open revolving drum and, while the drum is spinning rapidly, applying the spray and leaving to dry. The plastic coat may be removed by dipping into xylol if desired.
13. Label the dry film with India ink.
14. Place the sections in the holder supplied with the kit to keep the sections flat during microscopy.

These sections may be filed in special cabinets, stapled to records, suspended from racks, or filed individually after cutting a film strip apart. Before filing let the film strips dry for about 3 wk.

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*The 35 mm. film is Cronar, P-40B leader film, E. I. DuPont de Nemours & Co., Inc. The plastic spray is Krylon Plastic Spray, No. 1302, from Krylon, Inc., Norristown, Pa. The reel to carry the sections on film through staining solutions is the Nikor 35 mm. developing reel, made of stainless steel. It is available from photographic supply houses. A Slide-strip Kit containing all necessary apparatus is available from Carolina Biological Supply Co., Burlington, N. C.

Chapter 87

Staining solutions

ARAM A. KRAJIAN

INTRODUCTION

No satisfactory explanation has been advanced for the various staining effects of tissue and organisms except that these depend on chemical and physical factors. Advances in stain technology have resulted in a better understanding of the characteristics of tissue elements and structures, their affinity for numerous dyes, and with the aid of certain metallic and chemical mordants many specific staining methods have been developed.

The foundations for microscopic anatomic study were laid long before the staining methods were devised. In the early and midnineteenth century tissues were sectioned by hand in a crude manner, stained in various coloring solutions, and examined under cover slip preparations. Although the technics used at that time were crude and temporary, accurate observations of the material enabled the histologists to describe the arrangement of cells and tissue components in various organs of the body.

It was impossible, however, to study the cytologic elements and interlacing connective tissue and fibers of the central nervous system or structures of the special sense organs until the advent of staining methods. Cytology became a recognized science with the introduction of special stains which made it possible to color selectively certain elements of the cells such as chromosomes, mitochondria, and Golgi bodies.

The use of dyes in histologic microscopy began about 1850. Carmine stain was first introduced by botanists Göppert and Cohn[6] in 1849. Indigo, iodine, fuchsin, aniline red, aniline violet, and other dyes were used in simple solutions. Hematoxylin was first used by Waldeyer[7] to stain axis cylinders in aqueous solution. In 1865 Boehmer[8] combined hematoxylin with alum, thereby increasing its value as a histologic stain. Ehrlich,[9] Flemming,[10] and Unna[11] introduced both the aniline and hematoxylin series, opening the way for many more combination stains. About 1890 the differential stains were introduced as a result of combining various dyes and chemical reagents. Progress continued, and each year new important staining methods were announced. Mallory[12] developed his famous aniline blue stain for connective tissue; Heidenhain[13] developed his iron hematoxylin stain for protozoologic work; Levaditi[14] and Manouelian discovered a stain to demonstrate *Treponema pallidum* in tissue; Cajal[15] revolutionized the subject of neuropathology by his investigation and discovery of the value of silver nitrate for staining nerve fibers in the central nervous system.

Krajian developed more rapid and selective methods for the identification of such elements as myelin sheath, argentaffin cells, gram-positive and gram-negative organisms, parasites and inclusion bodies in tissue, urea crystals in brain tissue, reticular fibers, *T. pallidum* and other types of spiral organisms in tissue sections and smears, differentiation of carcinoma and sarcoma, elastic tissue, connective tissue, mucin, acid-fast organisms, zymogen granules, and others.

In the past half century great progress has been made and astonishing results accomplished in selective staining.

Staining solutions for specialized staining will be described with the specialized technic for which they are used.

HEMATOXYLIN STAINS

Hematoxylin is one of the oldest and most valuable histologic stains. Hematein, the active coloring agent, is formed by oxidation, which requires a number of days or weeks. In alum hematoxylin the selective staining power is due to the combination of hematein with alum. Mayer, Unna, and Harris have shown that it is possible to oxidize and ripen the alum hematoxylin by adding a little mercuric oxide or hydrogen peroxide. As alum hematoxylins become older they stain more quickly. Addition of 3 ml. glacial acetic acid to 95 ml. hematoxylin solution at the time of use helps sharpen the nuclear staining of the cells.

Harris hematoxylin:

Hematoxylin crystals	1 gm.
Alcohol, 95%	10 ml.
Ammonium or potassium alum	20 gm.
Distilled water	200 ml.

1. Dissolve the hematoxylin in the alcohol in a mortar with a pestle.
2. Dissolve the alum in water, with heat.
3. Add hematoxylin solution to alum solution.
4. Bring to a boil as quickly as possible.
5. Add 0.5 gm. mercuric oxide. The solution at once assumes a dark purple color. As soon as this occurs remove from the flame and cool as rapidly as possible in a bath of running or frequently changed cold water. When cool, the solution is ready for use.

Addition of 3 ml. glacial acetic acid to 95 ml. of solution at the time of use will increase its nuclear staining properties.

The stain is stable. It is one of the best routine histologic stains.

Filter before use.

Delafield hematoxylin:

1. Prepare a saturated solution of ammonium alum in distilled water.
2. Dissolve 4 gm. hematoxylin crystals in 25 ml. 95% alcohol.
3. Add 400 ml. saturated solution of ammonium alum.
4. Place in a Mason jar, with the cover on lightly, and put in the light and air for 1 or 2 wk.; then filter.
5. Add 100 ml. methyl alcohol, acetone-free, and 100 ml. glycerin.
6. Keep in a bottle exposed to the sunlight until ready for use. It usually takes at least 1 mo. for the hematoxylin to ripen.
7. **For quick ripening** add the methyl alcohol and glycerin to a freshly prepared and filtered hematoxylin solution; then add 400 mg. sodium iodate. The solution will darken and can be used immediately.

Delafield hematoxylin keeps well and is potent as long as it has a purplish cast.

Mallory phosphotungstic acid hematoxylin:

Hematoxylin or hematein ammonium	1 gm.
Phosphotungstic acid crystals	20 gm.
Distilled water	1000 ml.

1. Dissolve the hematoxylin in a small amount of water by heat.
2. Dissolve the phosphotungstic acid in the remaining water.
3. Allow the hematoxylin solution to cool and add it to the phosphotungstic acid solution.
4. If the solution stains weakly at first, it may be ripened by adding 5 ml. of freshly prepared 0.25% aqueous potassium permanganate for every 100 ml. stain, or it may be allowed to stand for several weeks until it ripens spontaneously. If hematoxylin is used instead of hematein ammonium, use 10 ml. permanganate solution/100 ml. When hematoxylin is used, addition of 0.177 gm. potassium permanganate/100 ml. solution may be substituted for the 0.25% $KMnO_4$.

Mallory phosphotungstic acid hematoxylin solution is especially useful to demonstrate fibrin as well as the fibroglia and myoglia fibrils of neuroglia. It also brings out the detail of mitotic figures, including spindles and centrosomes.

Heidenhain hematoxylin:

Formula 1:

Hematoxylin	1 gm.
Ethyl alcohol, 95%	10 ml.

1. Dissolve in a mortar and add 100 ml. of distilled water. Ripen several days.

Heidenhain hematoxylin, combined with iron and ammonium alum (see below), after suitable fixation, is used to demonstrate chromatin, nuclei, nuclear membrane, and centrosomes. The fixatives generally used are chrome salts and mercuric chloride.

Formula 2:

Hematoxylin	0.5 gm.
Ethyl alcohol, 95%	10 ml.

1. Dissolve in a mortar and add 100 ml. of distilled water. Ripen several days.

MacCallum hematoxylin:

Hematoxylin, 10% in absolute alcohol and ripened	2 ml.
Ferric chloride, 10% aqueous	3 ml.
Sodium iodide, 10% aqueous	5 ml.
Alcohol, 50%	50 ml.

Mix in the order indicated.

This is used for staining elastic tissue. The stain keeps 1 wk.

MacCallum hematoxylin—Krajian modification (Krajian iron hematoxylin):

Hematoxylin crystals	3 gm.
Ferric chloride	3 gm.
Ferric ammonium sulfate	3 gm.
Potassium iodide	3 gm.
Alcohol, 95%	25 ml.
Distilled water	25 ml.

1. Dissolve the hematoxylin in the alcohol.
2. Dissolve the other chemicals in the distilled water.
3. Mix the 2 solutions.

The resulting solution stains elastic fibers and myelin sheaths blue black, acts as a mordant in van Gieson stain, and stains bacteria.

This is used in the Krajian modification of MacCallum stain for elastic tissue.

Weigert-Pal hematoxylin:

Solution A:

Hematoxylin crystals	10 gm.
Absolute alcohol	100 ml.

1. Dissolve the hematoxylin in the alcohol and ripen for 2 or 3 wk.

Solution B:

Lithium carbonate, concentrated aqueous solution	7 ml.
Distilled water	93 ml.

1. Dissolve.
2. Mix 1 vol. solution A and 9 vol. solution B just before use.

This formula is used to stain myelin or medullary sheath.

Spielmeyer hematoxylin:

Ripened 10% hematoxylin in absolute alcohol	5 ml.
Distilled water	100 ml.

This is a myelin sheath stain.

Pal-Kulschitzky hematoxylin:

Ripened 10% hematoxylin in absolute alcohol	10 ml.
Acetic acid, 2%	90 ml.

The hematoxylin solution must be at least 6 mo. old.

This is a myelin sheath stain.

Fuller hematoxylin:

1. Dissolve 1 gm. hematoxylin in 90 ml. hot distilled water.
2. Cool and add 10 ml. glycerin and 10 ml. 95% alcohol.

This is used in staining acid-fast organisms.

Boehmer hematoxylin:

Solution A:

Hematoxylin crystals	1 gm.
Absolute alcohol	12 ml.

Solution B:

Alum	20 gm.
Warm distilled water	200 ml.

1. Filter when cool.
2. Mix the 2 solutions after 24 hr. Place in a wide-mouthed bottle and expose to the air for 8 days; then filter through filter paper.

Ehrlich hematoxylin:

1. Combine the following, dissolving the hematoxylin in the mixture of alcohol and acetic acid:

Hematoxylin crystals	2 gm.
Absolute alcohol	10 ml.
Glacial acetic acid	10 ml.

2. Add alcohol to glycerin as follows and mix:

Absolute alcohol	90 ml.
Glycerin	100 ml.

3. Add the hematoxylin solution to the alcohol-glycerin mixture.
4. Dissolve 10 gm. potassium alum in 100 ml. distilled water with heat.
5. Cool and add the alum solution to the hematoxylin–alcohol–acetic acid–glycerin mixture, stirring constantly.
6. Expose the solution to light and air to ripen for 4-6 wk.

If it stands in a warm place and is shaken occasionally, oxidation is facilitated, and the ripening process is aided materially. The ripe solution should have the color of port wine.

Mayer hematoxylin:

Distilled water	1000 ml.
Hematoxylin crystals	1 gm.
Ammonium or potassium alum	50 gm.
Sodium iodate	0.2 gm.
Citric acid	1 gm.
Chloral hydrate	50 gm.

1. Dissolve the hematoxylin in 1000 ml. distilled water that has been heated to 55°-60° C. and rotate until all the stain is in solution.
2. Add the alum and sodium iodate, taking care that the 0.2 gm. sodium iodate has been accurately weighed.
3. Rotate until the alum has dissolved; then add the citric acid and the chloral hydrate. The order of mixing must not be changed.
4. Allow the solution to stand overnight before use.

This solution is stable for 2 mo. if the staining time is gradually increased from an initial 6 min. to 8 min. after prolonged use. Occasional filtering may be necessary to remove accumulated cellular debris. It is used in the Pharr, Wood, and Traut modification of the Papanicolaou stain.

CARMINE STAINS

The active staining principle in the carmine solutions is carminic acid. All alkaline and acid solutions made with carmine owe their staining properties to carminic acid combined with aluminum.

Lithium carmine:

Carmine	2.5 gm.
Lithium carbonate, saturated aqueous	100 ml.
Thymol	1 crystal

The carmine dissolves at once in the cold solution. This solution should be carefully filtered, especially when used as a counterstain in the Gram-Weigert method.

Best carmine stain for glycogen:

Stock solution:

Carmine	2 gm.
Potassium carbonate	1 gm.
Potassium chloride	5 gm.
Distilled water	60 ml.

1. Boil several minutes, cool, and add:

Concentrated ammonium hydroxide	20 ml.

2. Keep this stock solution in well-stoppered bottles in a dark cool place. Filter before use. It keeps a month or less.

Differentiator:

Absolute methyl alcohol	40 ml.
Absolute ethyl alcohol	80 ml.
Distilled water	100 ml.

Mayer mucicarmine:

Carmine	1 gm.
Aluminum chloride	0.5 gm.
Distilled water	50 ml.

1. Heat in a Pyrex beaker for several minutes until completely dissolved.

2. Then add 50 ml. 95% alcohol and mix. The solution keeps well.

Mayer mucicarmine—Krajian modification:

Carmine	1 gm.
Aluminum chloride	0.5 gm.
Distilled water	25 ml.
Alcohol, 95%	25 ml.

1. Boil and dissolve the aluminum chloride in the distilled water.
2. Add the carmine slowly, stirring the mixture constantly for 3 min.
3. Cool, filter, and add 25 ml. 95% alcohol.

Azocarmine:

Azocarmine	1 gm.
Distilled water	100 ml.

1. Heat 20 min., cool, and filter.
2. Acidify with 1 ml. of glacial acetic acid.

Azocarmine-fuchsin: Mix 1 part of azocarmine stain (above) with an equal volume of 2% aqueous solution of acid fuchsin.

ANILINE DYES AND SOLUTIONS

Aniline dyes are sold as powders or in crystalline form. Most of the dyes keep well. There should be on hand saturated alcoholic solutions of certain dyes since the stains are fairly stable in such form and are ready for use when needed. This is particularly true of methylene blue, basic fuchsin, and methyl violet.

Aniline dyes are derived from aniline or toluidine or from both. They are salts with basic or acid properties. The basic dyes stain the cell nuclei and bacteria; acid dyes stain diffusely.

The basic dyes most often used are methylene blue, fuchsin, methyl violet, and safranin. The most commonly used acid dyes are eosin, picric acid, and fuchsin.

Loeffler methylene blue:

Saturated alcoholic solution of methylene blue	30 ml.
1:10,000 potassium hydroxide	100 ml.

1. Make 1:10,000 KOH by adding 2 drops 10% KOH to 100 ml. distilled water.

This is a useful aniline stain. It keeps well without losing much staining power.

Unna polychrome methylene blue:

Methylene blue	1 gm.
Potassium carbonate	1 gm.
Distilled water	100 ml.

1. Dilute 1:5 or 1:10 at the time of use.
2. The solution requires months for oxidation at room temperature. The time is greatly shortened if heat is applied.

This formula is extremely valuable as a stain for plasma cells and as a general stain combined with eosin, which should be used first. It is one of the older alkaline methylene blue formulas.

Acid polychrome methylene blue—Goodpasture:

Methylene blue	1 gm.
Potassium carbonate	1 gm.
Distilled water	400 ml.

1. Dissolve the ingredients and boil in a flask for 30 min. As the methylene blue is polychromed, most of it will precipitate.
2. Cool and add 3 ml. glacial acetic acid; shake thoroughly until the precipitate is dissolved.
3. Boil gently for 5 min. or until the solution is concentrated to a volume of 200 ml.
4. Cool in tap water.

The stain may be used immediately. It keeps indefinitely.

Modified Mallory aniline blue:

Aniline blue, water-soluble	0.5 gm.
Orange G	2 gm.
Oxalic acid	2 gm.
Distilled water	100 ml.

1. If necessary, heat to dissolve.

Mallory aniline blue–orange G–acetic acid solution:

Aniline blue, water-soluble	0.5 gm.
Acetic acid, glacial	8 ml.
Orange G	2 gm.
Distilled water	100 ml.

1. Heat and filter after cooling.
2. Dilute with as much water as there is stain.

Krajian aniline blue for elastic fibers:

Resorcinol	3 gm.
Aniline blue, water-soluble	1.5 gm.
Orange G	2.5 gm.
Phosphomolybdic acid	1 gm.
Distilled water	100 ml.

1. If necessary, heat to dissolve.

Borrel methylene blue:

1. Dissolve 1 gm. methylene blue in 100 ml. distilled water and heat in a water bath.
2. Dissolve 0.5 gm. silver nitrate in 100 ml. distilled water, placing the solution in a 500 ml. flask.
3. Dissolve 3 gm. sodium hydroxide in 100 ml. distilled water and add slowly to the silver solution, shaking the flask constantly. A brown precipitate of silver oxide is formed. Discontinue adding the sodium hydroxide solution as soon as no more precipitate is formed.
4. When the precipitate settles, discard the supernatant fluid, wash the precipitate by shaking in distilled water, and repeat the entire process several times until the distilled water is no longer alkaline to litmus paper.
5. Pour off the distilled water and add the 1% methylene blue solution.
6. Boil the mixture gently for 5 min. and cool. The solution becomes dark violet when cold. If still blue, boil further to oxidize the methylene blue. If it remains reddish, add a little more methylene blue until it becomes violet.
7. Filter and store in a glass-stoppered bottle.
8. To use, dilute 1 part stain with 5 parts distilled water.

Unna pyronine–methyl green:

Methyl green	0.15 gm.
Pyronine	0.25 gm.
Alcohol, 95%	2.5 ml.
Glycerin	20 ml.
Carbolic acid, 0.5% aqueous	100 ml.

1. Mix the alcohol and carbolic acid.
2. Dissolve the methyl green in the alcohol-phenol.

3. Mix the glycerin and distilled water.
4. Dissolve the pyronine in this mixture.
5. Mix the 2 solutions.

This solution is used principally in one of the chromatin methods.

Basic fuchsin solutions: A saturated alcoholic solution of basic fuchsin should be kept in stock.

Basic fuchsin	3.5 gm.
Alcohol, 95%	100 ml.

1. Grind in a mortar to dissolve.
2. Filter after 24 hr.

Basic fuchsin of superior quality, called "new fuchsin," is highly recommended for its deep staining of acid-fast organisms.

Ziehl-Neelsen carbolfuchsin:

Saturated alcoholic solution of basic fuchsin	10 ml.
Carbolic acid, 5% aqueous	90 ml.

1. Make the carbolic acid solution by adding 5 ml. melted carbolic acid crystals to 95 ml. distilled water. Shake.

This carbolfuchsin is quite powerful. It keeps well and stains well. Shake the bottle before use.

Krajian carbolfuchsin mixture for mucin stain:

Carbolfuchsin	15 drops
Acetic acid	3 drops
Formaldehyde, 40%	3 drops
Distilled water	2 ml.

The solution does not keep and therefore should be prepared fresh before use.

Fuller aniline fuchsin: Mix 16 ml. saturated alcoholic solution of basic fuchsin with 84 ml. aniline water.

Goodpasture fuchsin solution:

Formula 1:

Alcohol, 30%	100 ml.
Basic fuchsin	0.59 gm.
Aniline oil	1 ml.
Phenol crystals	1 ml.

Formula 2:

Alcohol, 20%	100 ml.
Pure phenol	1 ml.
Aniline oil	1 gm.
Basic fuchsin	0.5 gm.

The finely powdered or granular dye is easily dissolved, and the solution is ready for use.

Acid fuchsin solution: Keep a 1% aqueous solution of acid fuchsin on hand. Acid fuchsin is used in connective tissue stains, van Gieson formula.

Aniline acid fuchsin:

Acid fuchsin	2 gm.
Aniline water, 5%	10 ml.

Altman aniline fuchsin:

1. Prepare a saturated solution of aniline oil in distilled water by shaking the 2 together. Filter.
2. Add 10 gm. acid fuchsin to 100 ml. filtrate.
3. Use after 24 hr. It deteriorates in a month.

Mallory basic fuchsin:

Basic fuchsin	0.3 gm.
Alcohol, 95%	50 ml.
Distilled water	50 ml.

1. Dissolve the dye in the alcohol and add the water.
2. Filter after 24 hr.

Fuchsin-methylene blue:

Basic fuchsin	1 gm.
Methylene blue	0.5 gm.
Glycerin	50 ml.
Alcohol, 95%	50 ml.

1. Dissolve the fuchsin and methylene blue in alcohol, add the glycerin, and mix thoroughly.
2. Dilute for use, 1 part with 30 parts distilled water.

Fuchsin-methylene blue—Frothingham:

Saturated alcoholic solution of basic fuchsin	6 drops
Saturated alcoholic solution of methylene blue	4 drops
Distilled water	30 ml.

1. Add the dyes to the distilled water and mix.

Weigert iron-fuchsin stain for elastic tissue: This is also known as Weigert elastic tissue stain.

1. Mix 2 ml. melted phenol crystals and 1 gm. basic fuchsin; then dissolve in 100 ml. distilled water.
2. Bring to a boil; then gradually add 12.5 ml. 30% ferric chloride solution. A precipitate forms. Continue boiling at least 5 min. to render the precipitate more granular.
3. Cool and filter.
4. Wash the precipitate on filter paper with distilled water until no more color is seen. Dry the precipitate in a paraffin oven at 55° C. Keep the dry powder in a bottle as the stock dye.
5. To prepare the staining solution dissolve 0.75 gm. of the dry powder in 100 ml. 95% alcohol.
6. Boil in a water bath in a flask plugged with cotton wool for 20 min. Cool, filter, and add 2 ml. concentrated hydrochloric acid.

This is an excellent stain for demonstration of elastic fibers.

Methyl violet: A 1% aqueous methyl violet solution should be kept on hand. It keeps well.

It is used for staining bacteria and amyloid. With methyl violet, amyloid stains pink but fades after mounting.

Gentian violet: Gentian violet is a mixture of crystal violet, methyl violet, and dextrin. A product called "gentian violet improved" has been found satisfactory.

Sterling gentian violet:

Gentian violet improved	5 gm.
Alcohol, 95%	10 ml.
Aniline oil	2 ml.
Distilled water	88 ml.

1. Dissolve the aniline oil in the distilled water by shaking, and filter through filter paper. Add this to the gentian violet dissolved in alcohol.

The solution keeps well for several weeks or months and is highly recommended for demonstration of bacteria, fungi, and fibrin in tissue.

Carbol gentian violet:

Saturated alcoholic solution of gentian violet	10 ml.
Carbolic acid, 1%	90 ml.

1. Mix and filter after 24 hr.

Gentian violet saturates at 5 gm./100 ml. 95% alcohol.

Bensley neutral gentian violet:

1. Dissolve 1 gm. gentian violet in 25 ml. distilled water.
2. Dissolve 1 gm. orange G in 25 ml. distilled water.

3. Mix the 2 solutions and shake. A precipitate is formed.
4. Filter; wash the precipitate on the paper once with distilled water and dry.
5. Dissolve the dried precipitate in 25 ml. absolute alcohol. This is a stock solution.
6. For staining add a sufficient amount to 20% alcohol to give a deep violet color.

Kuhne crystal violet:

Saturated alcoholic solution of crystal violet	9 parts
Ammonium carbonate, 1% aqueous	1 part

1. Use absolute alcohol for making the saturated solution.
2. Mix just before use.

Eosin: Eosin is used principally as a counterstain following hematoxylin and at times methylene blue. It may be obtained as "eosin soluble in water" or as "eosin soluble in alcohol." It is used in strengths from 0.5% to 5%, at times dissolved in alcohol and at times in distilled water. Use eosin Y, water- and alcohol-soluble. When used before an aniline dye such as methylene blue, it should have a concentration of 5%. For counterstaining with hematoxylin a 1% aqueous solution is satisfactory. In the form of eosinol it gives excellent staining reactions, and eosinol is the recommended counterstain for routine sections.

0.5% Alcoholic eosin solution: Dissolve 0.5 gm. eosin Y in 100 ml. 95% alcohol.

1% Alcoholic eosin solution: Dissolve 1 gm. eosin Y in 100 ml. 95% alcohol.

2.5% Aqueous eosin solution: Dissolve 2.5 gm. eosin Y in 100 ml. distilled water. Place the eosin in a mortar, add a small amount of water, grind, add more water, and continue until all the eosin is in solution.

5% Eosin solution: Dissolve 5 gm. eosin Y (water- and alcohol-soluble) in 100 ml. distilled water or 95% alcohol.

Eosinol (for counterstaining, dehydrating, and clearing tissue at the same time): Krajian's method of hematoxylin and eosin staining consists in the dehydration of the section before counterstaining and the application of eosin as a solution in carbolxylol. Then, since the tissues pass afterward through xylol only, there is no opportunity for decolorization. Alcohol is saved because dehydration can be commenced in the stronger alcohol, discarding the lower gradations. The solution of eosin in carbolxylol, which constitutes the principal element of modification, is called eosinol.

1. Combine the following, dissolving the eosin in the water in a small beaker.

Eosin Y, water-soluble	5 gm.
Distilled water	10 ml.

2. Precipitate the eosin by adding:

Glacial acetic acid	10 ml.
Concentrated hydrochloric acid	2 ml.

3. Mix thoroughly with a glass rod. Incubate the resulting coagulum at 56° C. until all the water has evaporated. This may require several days.
4. Dissolve the dehydrated acid eosin in 10 ml. absolute alcohol mixed with 20 ml. acetone.
5. Stir with a glass rod for several minutes.
6. Cover the beaker tightly (aluminum foil may be used) and place in a paraffin oven (58°-60° C.) for 2 hr.
7. Let the undissolved portion, which is to be discarded, settle to the bottom of the container, 10 min. or more.
8. Remove the clear portion with a clean dry pipet and add it to 1500 ml. carbolxylol (1 part melted phenol crystals and 3 parts xylol).
9. To obtain the entire eosin yield, transfer solution remaining in the beaker to a test tube; then centrifuge and add whatever eosin solution (supernatant fluid) remains to the carbolxylol. Some precipitate may eventually form in the eosinol. Use only the clear supernatant fluid.

The solution keeps indefinitely. All glassware must be dry.

Because the various brands of powdered eosin do not have uniform staining power, it may be necessary to standardize the solution by staining control sections and adjusting the staining time or the strength of the solution.

Phloxine: Phloxine is used as a counterstain after hematoxylin. Some believe it to be superior to eosin. It stains more intensely, but differentiation is not as complete as with eosin stains.

A 1% solution is used for counterstains.

Picric acid solution: Keep a saturated solution of picric acid in stock. It is used most often in van Gieson stain and Bouin fluid.

Orcein solution: Orcein is a vegetable dye, which is used mainly for elastic tissue stain. It is soluble in alcohol. It is used in either neutral or acid alcoholic solutions.

Tincture of iodine (alcoholic solution): Tincture of iodine is used principally to dissolve the mercury precipitates that form in tissues fixed in mercuric chloride or other fluids containing bichloride, such as Zenker fluid.

Lugol solution:

Iodine	1 gm.
Potassium iodide	2 gm.
Distilled water	100 ml.

1. Rub up the iodine and potassium iodide in a mortar until thoroughly blended.
2. Add the water, a small amount at a time, until the iodine is completely dissolved.

Lugol solution is used as a test for glycogen, amyloid, and corpora amylacea.

Gram's iodine:

Iodine	1 gm.
Potassium iodide	2 gm.
Distilled water	300 ml.

1. Prepare as with Lugol solution.

This stain is used in the Gram-Weigert method of staining microorganisms and fibrin in tissue.

4% Aqueous Congo red: Dissolve 4 gm. Congo red in 100 ml. distilled water.

Krajian Congo red stain:

4% Aqueous Congo red	8 ml.
Glycerin, c.p.	2 ml.

Scharlach R (scarlet red)—Krajian modification:

Scharlach R	5 gm.
Acetone, pure	50 ml.
Alcohol, 70%	50 ml.
Benzoic acid	1 gm.

1. Dissolve the benzoic acid in the alcohol, then add the acetone, and saturate the dye in the combined solutions.

The stain is stable. After use it may be poured back into the bottle and used over and over. Filter before use. The benzoic acid intensifies the staining property of fats and prevents rapid deterioration of the stain.

Giemsa stain: The methods of making the stock and dilute Giemsa stain are given on pp. 1139 and 1140ff. The diluted stain must be prepared immediately before use. The stock stain is stable.*

Van Gieson stain:

Saturated aqueous solution of picric acid	100 ml.
Acid fuchsin, 1% aqueous . .	5 ml.

This solution keeps fairly well. If the stain weakens by long standing, restore by adding a few drops of fresh acid fuchsin.

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*Certified Giemsa stain made by Gradwohl Laboratory Supply Co., St. Louis, Mo., is recommended.

Chapter 88

Frozen sections

ARAM A. KRAJIAN

It is essential to eliminate as completely as possible all lost time and lost motion from laboratory methods. The specialty is already so overcrowded with procedures that any saving of time, especially with promise of increased exactitude, should be welcomed by the practicing clinical pathologist and laboratory technician.

The advantages of the frozen section method are (1) ease of manipulation, (2) saving of reagents, (3) lack of change in the normal structure of tissues, (4) definite saving of time, which allows for more rapid diagnosis and permits the technician more time for other procedures, and (5) less expense in preparation. Slides are permanent.

PREPARATION OF TISSUE FOR FROZEN SECTIONS

Blocks of tissue: In order that the fixing solution penetrate thoroughly, it is essential that the blocks be fairly regular and thin.

Fixing solution: The blocks should be immersed in 15% formalin for 24 hr. This kills bacteria and initiates the hardening and preservation of the tissue. Immediate fixation is essential.

Trimming blocks: After 24 hr. fixation the blocks should be trimmed to about 1.5-1.75 cm. square by 3-5 mm. thick and placed in 10% formalin (neutral) (p. 1641).

Rapid fixation: For rapid fixation, in cases in which one is sectioning tissue from biopsy at the operating table, drop the tissue in boiling 10% formalin for 30 sec. and then in dioxane for 30 sec., and freeze immediately. For rapid fixation of autopsy material use formalin that has been brought to the bubbling point in a test tube; then pour it on the tissue in a bottle, which reduces the temperature of the formalin to about 65° C. Allow it to remain in an oven at 56° C. 20-30 min. Shrinkage will be not more than 5%.

Washing: Have handy a beaker or glass dish about 6 in. in diameter and about 3 in. high. A 250 ml. beaker will suffice. Fill with tap water. Place the tissue blocks in the water for a short period only. This removes the formaldehyde which is detrimental to the skin and which could prevent the block from adhering to the holder. If the tissue is left in water for several hours, it becomes soft and good sections cannot be obtained.

Orienting the block: With forceps place the block on the block holder as follows:

Place a drop of water on the holder.

Place the edge of the block facing the knife parallel to the cutting edge, the smaller width of the block facing the cutting edge of the knife. If a capsule, skin, or mucous membrane is present, it must face the knife either on the shorter width or on the left on the longer edge so that the cut is made from the capsule into the tissue; otherwise the capsule may be torn off.

Freezing the block

Place the index finger on the block and turn on the CO_2 gas slightly for temporary holding. Too much gas is wasteful and also results in too rapid freezing.

Press down firmly but gently all around to fix the tissue to the holder and release more gas gently until the freezing line, which can be noted on the side of the block, has passed upward through about ½ of its depth. Even freezing is essential to prevent holes in the section. Any unfrozen portion will break during the cutting process. It is most important to press the block to the holder firmly, releasing the gas slowly.

Excess water may be removed with a towel while freezing. It is not necessary to use a continuous flow of gas.

Preparing to cut frozen section

Cut off the upper ⅓, that is, the unfrozen portion, and drop it back into the specimen bottle. Then shave down to establish a smooth, level surface on which to begin sectioning. If the block is small, it should be frozen throughout and sectioning begun by shaving off the top. If the tissue is too hard, it is likewise better to begin shaving from the top of the block; otherwise the block may be dislodged. If the surface is too soft, the section will have holes. The ideal is a hard, pliable surface so that a section cut therefrom will show no tendency to break as it piles up over the cutting edge of the knife.

If holes appear in the block while cutting, touch all parts of the cut surface gently with the fingers to warm it and melt it slightly; then refreeze. In this way the holes in the unfrozen parts will become filled with frozen moisture and there will be no fragmentary parts of the tissue.

Cutting frozen section

Operating microtome (Figs. 520 and 524): It is important that the cutting be done by placing the hand on the rim of the flywheel, turning it slowly and evenly to cut a smooth, regular section. If the handle of the flywheel is used and the cutting is done automatically and rapidly, the sections are cut too fast and tend to curl or break into shreads. Such speed does not allow sufficient time to adjust the tissue surface to the proper temperature and consistency; in other words, the slower procedure permits warming of the cut surface of the block, which alone is the controlling factor in procuring a pliable section, free from undulations. This pliability prevents disintegration of the section when placed in water.

On the average, 3 notches indicate that the section will be about 10μ thick and 1 notch indicates about 3μ, due to freezing and thawing action. However, variations in tissues cause variations in thickness of cut sections; e.g., if lung and uterus tissues are each cut at 3 notches, the latter will be close to only 2 notches thick because the tissue is firmer, offers greater resistance, and tends to raise the edge of the knife. Hence, it may be necessary at times to use 4 or 5 notches when cutting uterine tissue.

Uneven sections are usually the result of freezing the block too hard and applying too much speed and unnecessary pressure, especially on firmer tissues.

Removing section from knife: Wet the tip of the little or ring finger with water, and with the finger carefully remove the section from the edge of the knife, with a downward movement at about a 45° angle to the knife blade. Place the section in water. The tissue will float out on top of the water. Keep the knife edge dry. Water left on the edge thaws the tissue with which it comes in contact, causing a hole in succeeding sections. Place the sections in a large dish of water, about 3 in. deep, on a black background.

Variations in tissues:

Fatty or mucoid tissues: Cut fatty or mucoid tissues 15μ thick. Leave the section on the edge of the knife. Apply 2 or 3 drops of 95% alcohol to dissolve the fat and leave about 1 min.

Remove the section with the tip of the finger and place it in water, where the surface tension will cause the fatty and mucoid tissue to spread out.

If this procedure is not satisfactory, it is sometimes helpful to place the section in alcohol for 30-45 min. and then into water.

If difficulty is still encountered, place the entire block in equal parts of acetone and 95% alcohol for 24 hr., refix in 10% formalin, and proceed as above.

Aorta, intestine, and stomach: Cross sections are the rule. To orient the block of aorta tissue the intimal surface should be to the left. Tissues with serosal surface must be placed on the block with the serosal surface to the left. The block has but a small base and freezing surface; hence freeze ¾ of the block and begin shaving off the very top of the block for sections.

Transferring frozen sections to glass slide: Clean the slide by dipping in acid alcohol and wipe dry. The dish in which the sections are floated should

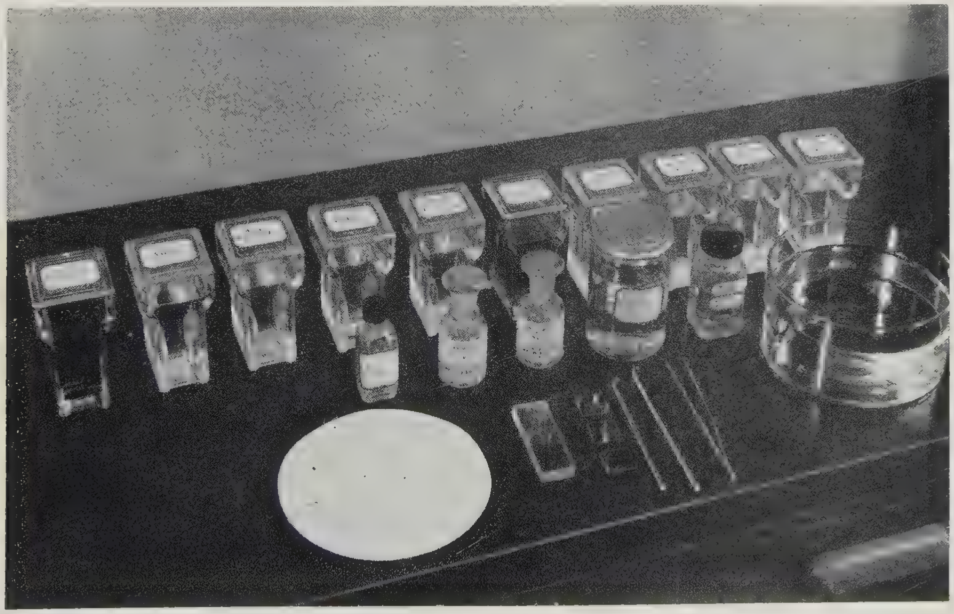

Fig. 528. Equipment and reagents for staining frozen sections.

be at least 7 cm. deep and 15 cm. in diameter. The operator should be seated so as to prevent any direct reflection from the slide to the eyes.

A glass rod, pointed or slightly bent, should be used to transfer the sections of delicate internal tissues to the slide. Teasing needles may be used for sections of firm tissues but not when one is staining for iron or using silver solutions for staining. See Fig. 528.

Anchor the section to the slide at about a 45° angle, with ¾ of the length of the slide under the water, in such a way that the edge of the water basin will afford support to either the slide or the fingers, thereby aiding in steadying the slide. By means of the glass rod pick up a good section, bringing it to the surface of the water immediately above the center of the slide.

When the section is centered, hold one corner at the upper and farthest side of the slide in place with the glass rod and slowly raise the slide out of water, always keeping the farthest edge of the slide slightly higher. Do not try to straighten out all folds under water. To eliminate any folds that may be present, place the edge of the slide with the folded portion of the section under water. With the aid of the water and the use of the glass rod, if necessary, the folds are readily eliminated. Care must be exercised in this procedure; first, keep the edge of the slide above the water wiped dry, and, second, keep the upper edge of the section above the water so that the section will not float away from the center of the slide.

After the folds have been removed tilt the slide against a staining dish at a 45° angle, face down, so that the water may partially drain. Do not permit it to dry, since drying may distort the section and shrink the cells. Time for draining depends upon the humidity of the atmosphere; this is usually from 15 sec. to 1 min. or more.

DEHYDRATING FROZEN SECTION

Holding the slide at a slight angle, drop at short range to prevent breaking the section 2 drops of anhydrous isopropanol or absolute alcohol on the section. Blow the breath over the section until the isopropanol or alcohol appears to have evaporated. The section becomes white. Blot twice with Whatman No. 2 filter paper.

Flood the slide with isopropanol or alcohol for 1-10 min.; then pour off the dehydrating agent, taking care not to disturb the section. Repeat if necessary. In fatty tissues, such as liver, brain, etc., fat is dissolved by alcohol and the section may tend to slip away from the slide. Under these circumstances blow over the surface of the section gently and remove the remaining alcohol by evaporation to keep the section on the slide.

Blot twice with several thicknesses of Whatman No. 2 filter paper.

Dip twice into a jar of thin celloidin (see below), in and out rapidly but carefully, taking care not to change the position of the slide.

Blow gently over the surface, again taking care not to change the position of the slide **until the celloidin is dry.** When thoroughly dry, place immediately into the staining solution. Mayer albumin fixative is not recommended.

Sections of mucoid tissue, after being properly drained, are dehydrated with isopropanol for 5 min.; then without blotting they are dipped in celloidin, dried by blowing over the surface, and stained. No filter paper is used to blot the sections because they are likely to stick to the paper.

Thick celloidin: Prepare a thick celloidin* as a stock solution by dissolving 30 gm. dry celloidin in 200 ml. of a mixture of equal parts of ether and absolute alcohol.

Shake the container several times a day for a week or longer, and stir the sediment with a heavy glass rod at least once a day until solution is complete.

Thin celloidin: Add about 7-8 ml. thick celloidin to a mixture of 45 ml. ether, 45 ml. absolute alcohol, and 10 ml. acetone in a dry container. When thoroughly dissolved, add 1 ml. saturated alcoholic solution of gum mastic to strengthen the adhesive qualities, make the sections smooth, and prevent rapid deterioration.

Medium celloidin: Mix equal parts of thick celloidin and ether-alcohol (equal parts) mixture.

PREPARATION OF LARGE FROZEN SECTIONS—CHRISTELLER METHOD

A sliding microtome† with large freezing table is used.

Slides are photographic plates 9 × 12 cm. and cover glasses† are 8½ × 11 cm. Staining dishes are rectangular-shaped museum jars. There should be about a dozen of these. Tissues should be formaldehyde fixed.

Follow the procedure described in detail for routine frozen sections.

ROUTINE STAINING OF FROZEN SECTIONS

Reagents:

1. Harris hematoxylin. See p. 1658.
2. Acid alcohol.
    - (a) Add 1 ml. concentrated hydrochloric acid to 100 ml. 70% ethyl alcohol.
3. Ammonia-isopropanol.
    - (a) Add 5 ml. ammonium hydroxide, c.p., to 100 ml. anhydrous isopropanol.
    - (b) If the ammonia volatilizes off the solution during use, replace with fresh ammonium hydroxide.
4. Eosinol. Refer to p. 1662.
5. Carbolxylol. See p. 1655.

Procedure:

1. Keep solutions in staining jars in proper order. Have on hand a drop bottle containing anhydrous isopropanol.
2. Put the slide (section mounted under celloidin, see above) in Harris hematoxylin for 3-10 min., or for rapid work dip in and out carefully about 50 times or until the section is the proper color.
3. Place in tap water until blue (4 min.).

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*Celloidin is that furnished by DuPont or Schering, or Parlodion made by Mallinckrodt Chemical Works, St. Louis, Mo.

†E. Leitz, Inc., New York, N. Y.

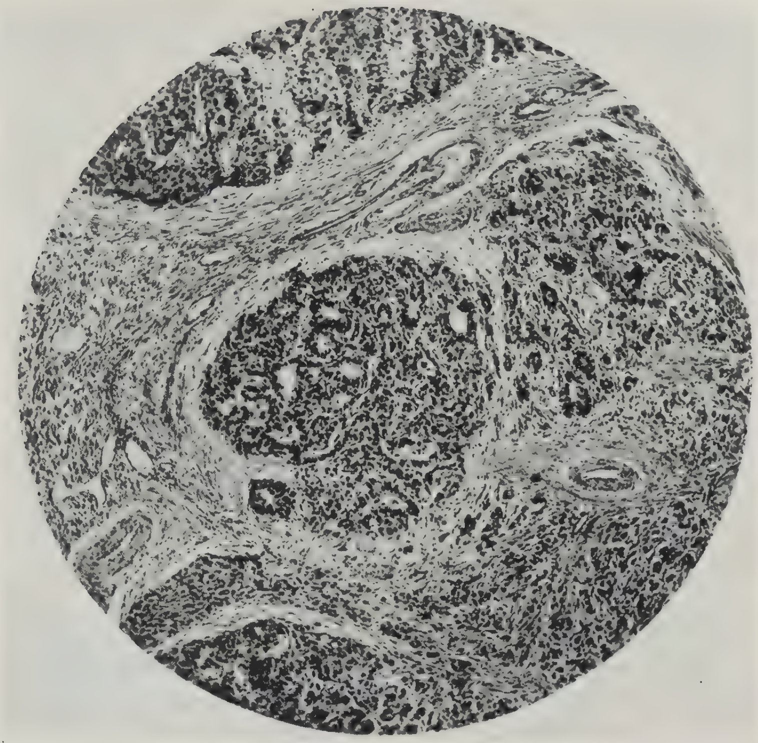

Fig. 529. Cirrhosis of liver. Hematoxylin-eosinol stain. Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

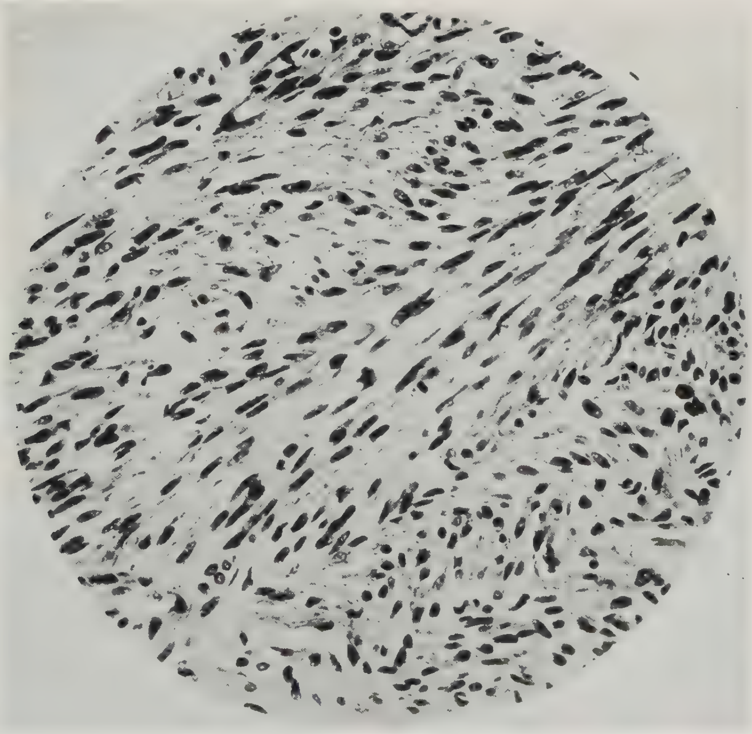

Fig. 530. Fibroma of uterus. Hematoxylin-eosinol stain. Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

4. Decolorize in acid alcohol by dipping the slide in and out of the jar, turning for even decolorization, until no more salmon pink solution runs off the tissue. Be sure to decolorize thoroughly, but do not overdecolorize.
5. Dip in distilled water to stop the decolorizing.
6. Place slide in ammonia-isopropanol until every part of the tissue is a good blue color. If desired, examine under the low power of the microscope to see if the nuclei are a good blue. Do not rush this step.
7. Pour anhydrous isopropanol over the slide (allowing it to flow into the ammonia-isopropanol jar to save it) to dehydrate the tissue. Several drops are sufficient, if repeated twice. Remove excess liquid from the back of the slide.
8. Dip in and out of eosinol several times, turning the slide for even staining and watching the staining in the air over a white background until the section is stained evenly red. This requires 10-30 sec. or more, depending on the strength of the eosinol.
9. Place in carbolxylol for 3-4 min. or dip in and out of carbolxylol until clear (about 30 sec.).
10. Drain and place for 1 or 2 min. in the first xylol or dip in and out of the xylol for several seconds.
11. Place in the second xylol for 2 min., then into the third xylol to clear.
12. Mount in neutral gum damar. Do not allow the xylol to dry before mounting.

SPECIAL FACTS ABOUT THE RAPID FROZEN SECTION METHOD

In all cases in which sections are transferred from one solution to another be sure to drain off as much of the solution as possible, especially with carbolxylol. Carrying of carbolic acid into xylols will cause fading of nuclear stains.

If the sections occasionally fall off because of swelling, flood them with isopropanol for 2-5 min., pour off, blow section dry, blot, dip twice in celloidin, and resume the process from that point on. This may occur with sections of liver, brain, cartilage, or spleen.

If sections do not clear properly, the isopropanol is not anhydrous, the carbolxylol has been used too long, or the tissue has not been properly dehydrated. Try new solutions.

If the sections are cut unevenly, the microtome may be at fault or the section was frozen too hard.

If a section has been recelloidined, it must be dehydrated with isopropanol before placing in xylol.

When gum mastic is old or in solution, it deteriorates. It is necessary in such cases to use more gum mastic than the method calls for.

If the celloidin covering the section takes the stain during the staining process, the stain may be removed by dipping the slide back into thin celloidin until clear again.

Always be sure the celloidin has been blown dry before staining, to prevent artifacts. The celloidin must completely penetrate the tissue before stain is applied; otherwise the cells will not take the stain.

Air bubbles in the section form because the section is too thick, there is not sufficient gum damar, the gum is too thin, or the cover slip may not be properly laid down.

Hard tissues such as uterus and others of fibrous structure may be softened by the application of several drops of dioxane at the time of cutting. Then refreeze. If the block is still too hard, the dioxane may be applied twice again. Never apply dioxane more than 3 times.

Soft tissues such as gelatinous carcinoma of lung tissue, if soaked in dioxane 15-30 min. before cutting, give good consistency.

Striated muscle fibers are best shown with the spirochete stain.

If sections show shrinkage, it is usually caused by drying of the sections before they are dehydrated and celloidined. Watch every section very carefully during the draining process, and if any one is even slightly dried, dip that portion back in water and begin the dehydration immediately.

If the outside walls of appendices turn over, it may be due to the drying of the specimen before it was fixed, and nothing can be done about it.

If the hematoxylin-eosinol stain is satisfactory, the nuclei will be clear under high power, the blues will be blue and not purple or lavender, while the eosin will be pink or red but not lavender or purple. If the blues have a purple cast, the section was not properly decolorized in acid alcohol nor the blue brought out properly in ammonia-isopropanol.

It is necessary occasionally to add more ammonium hydroxide to the ammonia-isopropanol to replace that lost by volatilization.

If the hematoxylin develops a sediment, pour off the supernatant liquid and continue to use it. A scum may be dissolved with a little glacial acetic acid.

If the celloidin peels off the slide or falls away from the tissue, this may be caused by using celloidin that is too old or that is contaminated with extraneous material. Make new celloidin. Or it may be due to the fact that some of the solvent has evaporated, resulting in a thickening of the celloidin. Either thin the celloidin with a mixture of alcohol, acetone, and ether in proper proportion or, better, make fresh thin celloidin.

If the absolute alcohol or anhydrous isopropanol dissolves the celloidin and loosens the section, blot and then dip again in celloidin as previously.

Do not allow the section to dry at any step. If the section becomes dry, dip it back into the solution from which it has just been taken.

METHOD OF REMOVING WRINKLES FROM CREASED FROZEN SECTIONS

Sometimes sections of brain, liver, and spleen are creased when submitted to clearing in carbolxylol or in fresh xylol. To flatten them, blot the section thoroughly, dip in thin celloidin twice, place in carbolxylol for 1 min., blot thoroughly in filter paper, clear in xylol in the usual manner, and mount in gum damar.

RAPID FROZEN SECTIONS AT THE OPERATING TABLE—PERMANENT MOUNTS (KRAJIAN)

Tissues or biopsies removed during surgery are brought to the laboratory to be examined for malignancy in cases in which rapid diagnosis is of prime importance.

1. Place thin pieces of tissue in a 50 ml. Pyrex tube containing about 30 ml. 10% formalin and boil immediately over a free flame for 15-30 sec.
2. Pour off the formalin, wash the tissue thoroughly in tap water, and make sections in the usual manner on the freezing microtome.

If desired, instead of washing the blocks of tissue after boiling in formalin, drop in dioxane for ½ min. and freeze and cut sections directly from the dioxane.

3. Place on slides.
4. Add a few drops of isopropanol (anhydrous) from a drop bottle and blow dry. Repeat and let evaporate.
5. Cover the slide with isopropanol for about 30 sec., drain off excess, and blot between several layers of Whatman No. 1 filter paper.
6. Dip into thin celloidin (p. 1666) twice. Wipe the back of the slide dry. Blow the breath over the surface of the slide to dry the celloidin. **Do not change the direction in which the slide is held during the celloidining process until the celloidin is dry.**
7. Have all reagents in staining jars.
8. Dip in and out of Harris hematoxylin about 50 times.
9. Wash in tap water for a few seconds.
10. Decolorize in 1% acid alcohol as in the routine method.
11. Dip in tap water to stop decolorizing and place immediately in the ammonia-isopropanol mixture until blue (p. 1666).
12. Drop a few drops of isopropanol over the surface of the slide to dehydrate it, catching the isopropanol in the jar of ammonia-isopropanol mixture.
13. Dip in and out of eosinol several times until the tissue is a good pink color. This requires only a few seconds.
14. Dip in and out of carbolxylol to clarify. About 20-30 dips usually suffice.
15. Dip in xylol for a few seconds, about 30-50 times.
16. Repeat in 2 more changes of xylol.
17. Remove excess celloidin from around the tissue and mount in gum damar.
18. If the specimen becomes elevated on the slide at any time, dip in celloidin again, then back into xylol, and proceed.

EMBEDDING METHOD FOR FROZEN SECTIONS

Embedding methods for frozen sections are intended for fragmented and loose-textured tissues, such as curettings, brain abscesses, and body fluids.

Embedding mass:

1. Mix 10 gm. powdered gelatin and 3 gm. Bacto-agar thoroughly.
2. Dissolve in 100 ml. boiling water containing 0.5 ml. liquefied phenol. This keeps well in a stoppered bottle.

To embed:

1. Fix the tissue in 10% formalin.
2. Liquefy the mass in a boiling water bath.
3. Dry the tissue with a towel, place and arrange it in a paper box containing the liquefied mass, and incubate at 50° C. for 2 hr. to allow penetration.
4. Allow the pool to solidify in a cool place or in a refrigerator for 2 hr. or longer.
5. Remove the blocks and trim.
6. Fix the block in 10% formalin 3 hr. or longer.
7. Make frozen sections, mount on a slide under celloidin in the usual manner, and stain as for frozen sections.

SERUMIZING METHOD FOR LOOSE-TEXTURED TISSUES—KRAJIAN

One of the principal objections to the use of frozen section methods has been the impossibility of securing satisfactory sections of fragmentary and loose-textured tissues, such as curettings, brain abscesses, and body fluids. Krajian[16] has overcome this objection by applying his serumizing method as follows:

Fix in 10% formalin in the usual manner for 24 hr., or longer if necessary. Trim the blocks. Wash several times in tap water to remove the formaldehyde. Place the tissue in a small Stender dish or specimen bottle containing uncontaminated human or animal serum. Fresh serum obtained from the day's serologic specimens is ideal for this purpose. The serum should completely cover the tissue. Cover the dish or bottle tightly and put in a warm place overnight. The top of the paraffin oven is a good place for it. Cover any floating tissues such as lung or fatty material with absorbent cotton in order to keep all parts submerged in the serum.

The following morning decant the excess serum. Without washing add sufficient dioxane to cover the blocks. Allow it to stand for 3-5 hr. or until the serum is completely coagulated. Fix in 10% cold formalin overnight or in hot formalin in a paraffin oven at 56° C. for 2 hr. Immediately freeze on the freezing microtome with carbon dioxide gas and cut, stain, and mount sections in the usual manner.

Since dioxane will eventually shrink the tissues, they cannot be kept in this solution indefinitely. Keep the blocks in 10% formaldehyde for permanent storage.

For emergency examination use very thin blocks of formaldehyde-fixed tissues. Place in serum in a paraffin oven at 56° C. for 1-2 hr.; then coagulate in dioxane for 1 hr. Then freeze, cut, stain, and mount.[17]

This method has been used in our laboratories frequently. It causes very little shrinkage and no curling or shriveling of sections such as other embedding processes intended for frozen sections. This serumizing process makes hard tissues, such as bone and uterus, soft and pliable, giving better sections.

Store the serum in small bottles and preserve by the addition of 2 ml. commercial 40% formaldehyde to 50 ml. of the fluid. This keeps well at room temperature indefinitely without contamination.

THE MICROTOME-CRYOSTAT*

The cryostat is a "cold-knife" refrigerated microtome for the preparation of frozen sections. It provides a rapid means of preparing large, thin, often unwrinkled sections of single or multiple pieces of fresh frozen tissue. It also permits rapid preparation of biopsies for surgical pathology. Like other instruments, proper manipulative skill must be developed by practice before the cryostat can be operated efficiently.

Approximately 4 hr. are needed for the microtome-cryostat to reach the required temperature. Before connecting the unit to the electrical circuit, lubricate all moving surfaces with the arctic oil provided. If the microtome becomes difficult to operate because of insufficient lubrication, place a few drops of alcohol on the moving surfaces. The technician should practice manipulation of the microtome and accessories before proceeding to refrigeration.

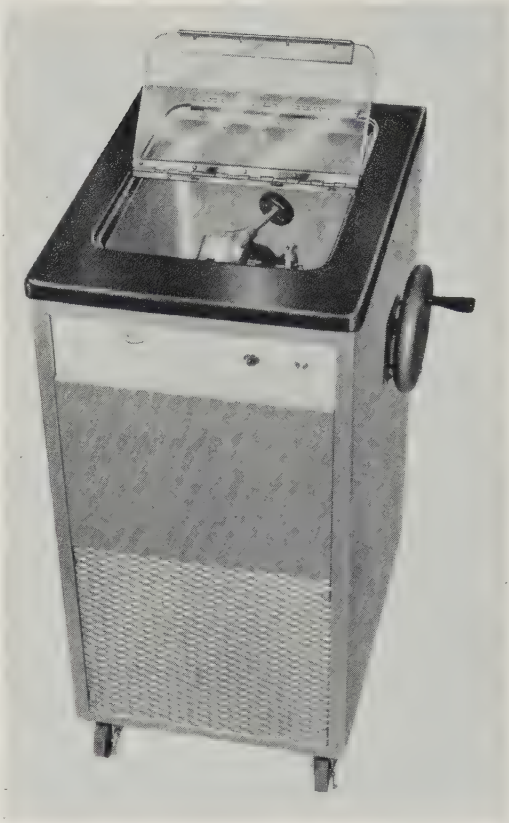

Fig. 531. Harris-International microtome cryostat, model CT. (Courtesy Arthur H. Thomas Co., Philadelphia, Pa.)

Place the microtome knife in an open knife box so that it may reach the equivalent temperature of the apparatus. The temperature of the knife should not be higher than −5° C., and the angle ordinarily used is approximately 30°. Too shallow an angle results in lack of sectioning, whereas too steep an angle gives rise to compression or splintering of the tissue if soft proteinaceous tissues are cut at too low a temperature.

Cutting technic:

1. Protect the hands from the cold with cotton or canvas household gloves.
2. Set the cold knife at approximately a 30° angle.
3. Set the thickness scale of the microtome. Each division on the scale is equal to 2μ.
4. Close the transparent top cover until subsequent steps have been carried out.
5. Select unfixed, previously refrigerated or fresh tissue blocks. The blocks should be several mm. deep to keep the knife edge clear of the metal object holder, and they must be cut so as to have parallel flat top and bottom surfaces. If minute or shallow tissues are presented for section, these may be supported on a thick slice of fixed, soft tissue.
6. Fill the ring cavities of the object holder with

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*Information on the model CT cryostat has been furnished by the International Equipment Co., Needham Heights, Mass., from the Handbook on the microtome-cryostat by John R. Baker, Director of Histology and Histochemistry, Bio-Research Consultants, Inc.

distilled water from a plastic washing bottle or a dropper.

7. Gently place the tissue on the wet holder, hold it in place with forceps, without squeezing, add a wall of water to surround the tissue, and freeze with the CO_2 attachment, with dry ice, or with Freon aerosol. The tissue adheres to the holder as the water turns to ice.
8. If the tissue needs support, add water around it to build a supporting wall of ice.

When the tissue is frozen sufficiently, the entire block becomes opaque. This is an extremely rapid operation. If the tissue is frozen in the cryostat chamber, ice crystal artifacts and desiccation of the tissue may occur. In emergencies, cover the tissue and holder with Saranwrap to prevent undue loss of moisture due to slow freezing.

9. Transfer the frozen tissue and object holder to the microtome head, which should be in the upper position with the drive wheel locked.
10. Tighten the rubber-covered head clamp sufficiently to hold the object holder firm.
11. Pull back the knife-holding clamp so that there is 1/4 in. clearance between the subsequent down travel of the block and the knife.
12. Release the lock of the drive wheel and bring the tissue block close to the knife; then adjust the specimen holder and tissue so that its travel is approximately plane to the knife edge.
13. Bring the knife to within 1-2 mm. of the tissue and make the final plane adjustment of the block to the knife, which must be precisely parallel. This adjustment is critical.
14. Bring the tissue face almost into contact with the knife edge, release the ratchet from the micrometer wheel at the rear of the microtome, advance the wheel clockwise by hand, a fraction of a turn, until the tissue begins to section, and continue trimming off the face of the tissue until the desired cutting plane is reached.
15. Return the ratchet to the teeth of the micrometer wheel.
16. Clean the knife entirely free of tissue and condensation debris.
17. Turn the drive wheel slowly until the leading edge of the section begins to cut.
18. With a red sable-hair water color brush, size 6 or smaller, gently stroke the section onto the microtome blade as it makes its downward travel from continued slow movement of the drive wheel and smooth and flatten the section if necessary.
19. Transfer the section to a "warm" slide or a slide at cryostat temperature, depending on the subsequent technic. Brush the knife clean and dry it between each section cut.

Mounting sections on "warm slides": Place a clean slide, at room temperature, a fraction of an inch over the frozen section on the cold knife. The section is attracted directly to the warm slide and transfers itself from the cold knife to the slide. Direct pressure causes distortion or stretching and is to be avoided. Once mounted on a warm slide, the sections are ready for immediate staining or cold storage.

Mounting sections on "cold slides": Place clean slides in a small, open-slotted box within the cryostat chamber until they reach cryostat temperature, which requires only a few minutes.

Place the frozen section on the cold slide, smooth and flatten with the brush, and then remove the slide to room temperature.

Place a finger under the section on the undersurface of the slide. The slight warmth causes the section to flatten out and adhere to the slide. Unless the section is brushed out absolutely flat prior to thawing, there is possibility of trapping minute air bubbles under the tissue. Once it has been thawed and adheres to the slide, continue as previously.

Fixation of the section: The cryostat permits rapid fixation. A 2-10 min. immersion in chilled (+5° C.) fixative is sufficient for most purposes. The recommended fixatives are acetone, acetic-alcohol, alcohol-ether (equal parts), formal-alcohol (formalin 10, absolute alcohol 80, distilled water 10), Carnoy, and neutral 4% formaldehyde (10% formalin). For the study of fats, only the formalin fixative may be used. In enzyme histochemistry, where fixation is permitted, 2 min. fixation is often sufficient.

For rapid biopsy technic apply fixative at room temperature for 30-60 sec. A rapid and suitable fixative consists of 1 part formalin to 9 parts ethyl alcohol.

Staining the section:

1. Rinse briefly in 70% alcohol, then in 50% alcohol, and then in distilled water.
2. Stain for 1 or 2 min. in well-ripened Ehrlich hematoxylin (p. 1659).
3. Rinse in distilled water.
4. Immerse in 0.1% sodium borate for a few seconds until the tissue turns blue.
5. Rinse in distilled water 30 sec.
6. Stain in 1% eosin for 10 sec.
7. Dehydrate through 3 changes of ethyl alcohol, 5 sec. each; then transfer to equal parts of absolute alcohol and xylol.
8. Clear in xylol and mount.

Another method is to stain briefly (a few seconds) in 1% aqueous methylene or toluidine blue, or in Paragon frozen section stain, rinse rapidly in water, dehydrate rapidly, and clear and mount. The section may be viewed directly from water if desired.

Chapter 89

Paraffin sections

ARAM A. KRAJIAN

GENERAL FACTS ON PREPARATION AND EMBEDDING OF PARAFFIN SECTIONS

Paraffin sectioning differs from frozen section methods in that, with this method, the water in the tissue is replaced in the section by paraffin and then thin sections are cut. In order to remove the water and entirely replace it with paraffin, the tissue, after proper fixation, must first be dehydrated. This is accomplished by carrying it through increasing strengths of alcohol or isopropanol, beginning usually with 70% strength. The water is gradually replaced with the dehydrating agent until all the water has been removed and 100% (absolute) alcohol or anhydrous isopropanol is present in its place. The alcohol is then replaced by xylol, which clarifies the tissue; the xylol permits the penetration of the tissue by paraffin, which then replaces the xylol. This entire process of dehydrating, clarifying, and embedding may require several days, at the end of which time the tissue is embedded in pure paraffin, then cut into sections of appropriate thickness, and stained.

Before the sections can be stained they must first be affixed to the slide. The paraffin must then be removed, which is accomplished by the use of xylol. The section then passes through gradually decreasing strengths of alcohol or isopropanol, which removes the xylol and eventually restores the original water content of the tissue. The section is now ready to be stained.

Paraffin embedding is useful when very thin sections are desired. For good results the tissue blocks should be small, preferably not more than 2 or 3 mm. thick. The time of dehydration varies according to the size and consistency of the tissue. The amount of alcohol used should be much greater than the bulk of the object. The alcohols should be kept in pint or quart bottles. They are prepared by diluting 95% alcohol or isopropanol to desired strength.

Staining processes are quite similar to each other. The section is first submitted to hematoxylin stain, decolorized in an acid to remove the dye from all portions of the tissue except the nuclei, the blue color restored with an alkali, and then the section counterstained with a contrasting color (red, of the eosin); the nuclei remain blue; other portions take the eosin dye.

The tissue must again be dehydrated since it is to be clarified and mounted in a material immiscible with water. Alcohols of increasing strengths are used, followed by xylol as a clearing agent, after which the tissues are mounted under a cover glass. By the use of eosinol (p. 1662), the dehydration, counterstaining, and clarification process are combined in one step.

Preservation of tissue

Tissues should be kept in alcohol fixatives not longer than the time specified in the particular method. If they must be kept some time before embedding in paraffin, preserve them in 70% alcohol. Lower alcohol concentrations macerate tissues; higher grades harden them excessively. Prolonged preservation of specimens in alcohol affects the staining property of the nuclei. Formalin-fixed tissues should be kept in 10% neutral formalin.

The **formalin-alcohol-paraffin method** can

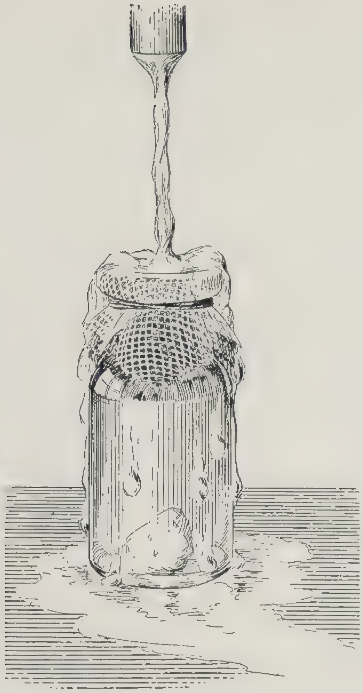

Fig. 532. Washing tissues.

be used on all tissues. Formalin kills, fixes, and hardens the tissue, so that the tissue remains intact, undergoing no further change. The formalin does not shrink the tissue nor expand it. It penetrates all parts equally well, including fat.

After fixation, the tissue should be washed in running water or placed in a bottle of water, changing the water frequently. All formalin must be removed. The underlying principle by which penetration of the tissue is accomplished by the various solutions is the principle of osmosis. The various steps must not be taken too rapidly.

Identification tags

The bottle containing the specimen should be labeled with a tag around the neck; it is safer to place a small pencil-marked label inside the vessel containing the tissue. This prevents confusion and mistakes if many different pieces of specimens are handled at the same time. The tag around the neck of the bottle is used not only for identification but also for the record of the solutions through which the objects are being passed.

Clearing process

The reagents employed in clearing tissue are chloroform, benzine, xylol, cedar oil, dioxane, and butyl alcohol. To clarify, the absolute alcohol is discarded and a large volume of clearing reagent is added to the bottle containing the specimen. For a large object the clearing solution should be changed at least once. The time required for clearing varies according to the size and consistency of the tissue and the particular clearing reagent used. Xylol rapidly removes alcohol and clears the tissue. Tissues should be kept in xylol for a minimum time only, since prolonged treatment causes the tissue to become brittle.

Chloroform enables the paraffin in the mixture to partially infiltrate the tissue and then rapidly evaporates from the pure paraffin bath. Its hardening effect is very much less than that of xylol.

Benzine clears quickly, makes the tissue more transparent, and evaporates rapidly from the paraffin bath without much hardening.

Cedar oil does not harden the tissue even by prolonged immersion. It clears from 95% alcohol but has the disadvantage of slow penetration and subsequent difficulty of elimination in the paraffin bath.

Paraffin embedding process

An electrically controlled paraffin oven is needed. The temperature should be maintained slightly above the melting point of the paraffin used. A temperature of 56°-58° C. suffices for most purposes.

Paraffins with a high melting point are hard when allowed to cool; those with a low melting point are soft. (1) For hard objects it is essential to use hard paraffin in order to support the tissues during section cutting. A lower melting point paraffin is used for soft objects. (2) The degree of hardness of the paraffin block containing the object depends on the surrounding temperature. In summer or in tropical climates it is necessary to use high melting point paraffin to obtain the correct consistency for sectioning. For ordinary work in temperate climates paraffin with a 48°-50° C. melting point is satisfactory. For hard objects or for section cutting in warm weather, it is necessary to use paraffin of 55° C. melting point.

Paraffin with different melting points is available from a number of laboratory supply houses.* Refer to p. 1674.

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*Pure paraffin is made by Arthur H. Thomas Co., Philadelphia, Pa., Matheson-Coleman & Bell Division, the Matheson Co., Inc., E. Rutherford, N. J., or Fisher Scientific Co., New York, N. Y.

Infiltration of paraffin

After tissues have been dehydrated and thoroughly cleared it is necessary to remove the clearing reagent and infiltrate with paraffin. This is done by adding an equal amount of melted paraffin to the clearing reagent and placing it in a paraffin oven. As the clearing medium warms, it dissolves the paraffin and permeates the objects with the mixture. When infiltration is completed, the clearing and paraffin mixture are discarded, and the tissues are soaked in melted paraffin, in a paraffin oven. The paraffin is changed at least once. The duration of time in the paraffin baths depends on the size and thickness of the object, more time being required to penetrate a large, dense object than a thin, loose-textured tissue.

The object must be kept in the oven just long enough for complete paraffin infiltration. Any traces of clearing reagents remaining in the tissues or the failure of the paraffin to penetrate the tissues indicates insufficient time in the paraffin bath and prevents proper section cutting. Prolonged treatment in the paraffin oven must be avoided because this causes shrinkage and hardening of tissue and makes section cutting very difficult. The complete removal of the clearing reagent from the object requires a sufficient number of changes of paraffin.

Casting or embedding process

The tissues are first placed in molds of the correct size filled with melted paraffin, the side of the tissue which is to be cut facing the bottom of the mold. The paraffin mold is then allowed to harden by cooling.

There are several types of molds for paraffin, such as the metal boxes made with pairs of variously sized L-shaped metal pieces and hand-folded paper boxes. The L-shaped metal pieces are set one against the other on a glass or metal plate. The shape and size of the block can be varied with the shape and size of the object.

Procedure for embedding

1. Fill the boxes with melted paraffin.
2. Using warmed forceps, place the tissue in the bottom of the paraffin with the side to be cut facing the bottom of the box.
3. Label the boxes with the case number.
4. Either set the box on a wet towel or gently immerse it in a basin of ice water so as to cool the paraffin rapidly. Or permit the paraffin to harden at room temperature.
5. Do not attempt to trim the block before the paraffin has completely set. Small blocks of tissue should be left in water 15 min. or longer to ensure complete solidification of the paraffin. Large blocks require 30 min. or more.

Making the paper box or "boat"

Make the box from paper or aluminum foil about 0.1 mm. thick. Fold the paper according to the diagrams in Figs. 533 and 534. If aluminum foil is preferred, simply fold it around a small wooden block with a rectangular surface.

Paraffin used for embedding tissues

Special paraffin is manufactured for embedding purposes. Different samples of paraffin have different melting points and also different "plastic points."* Paraffin is a mixture of hydrocarbons that solidifies into char-

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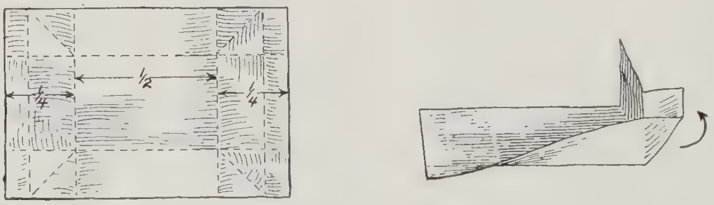
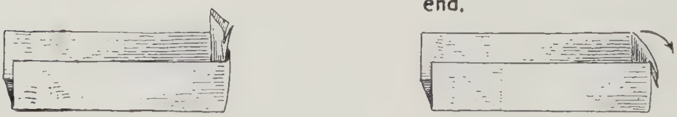
*We are indebted to Dr. O. W. Richards for the following material from his pamphlet "The effective use and proper care of the microtome," American Optical Co., Scientific Instruments Division, Buffalo, N. Y.



①Crease on dotted lines

② Fold up one end and one side. Fold projecting flap against the end.



③Repeat with adjoining corner.

④Fold over and crease the projecting end-flap to lock the corner joints.

Fig. 533. Method of making "boats" for embedding paraffin sections.

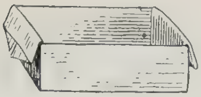


Fig. 534. Completed "boat," ready for use.

acteristic types of crystals, varying to some degree with the proportion of harder and softer hydrocarbons present. The peripheral crystals are oriented with respect to the cooling surface while the center of the mass forms a meshwork (Dempster, 1941). The "plastic point" is the lowest temperature at which permanent deformation may be made without fracture. A paraffin with low plastic point appears more translucent, is less brittle, but compresses more in sectioning. The hardness of paraffin depends on its plastic point which lies a few, but variable number of, degrees below the melting point. Consequently, the plastic point or, more roughly, the melting point of the paraffin has to be adapted to the temperature of the room in which the sectioning is being done.[18] Warm rooms require a higher melting point paraffin than do colder laboratories. The alternative is to condition the paraffin by the addition of various materials. Bayberry wax, rubber, and other waxes may be added to paraffin to improve its plasticity and cutting qualities.

In addition to matching the hardness of the paraffin to the temperature at which it is to be sectioned, it is necessary to match the hardness of the paraffin to the hardness of the tissue. The hardness of the tissue depends upon the preparatory treatment and its structure. The actual process of sectioning leads to distortion so that the sections will be slightly thicker and a little shorter than the block itself. This distortion results from the nature of the paraffin, the tissue, and the action of the knife. Large crystals are sectioned or pushed apart by the wedging of the knife, especially when the paraffin is brittle and has a high plastic point. This upsetting of crystals gives the velvety appearance to the topside of the section.

When the crystals are not of proper size to fit closely to the tissue, they cannot support the tissue adequately, and local deformation will occur, giving a section which cannot be completely flattened out. Small folds show when the thin parts are smaller than the size of the paraffin crystals and compress less than the paraffin. The crystals show clearly in polarized light. All microtomists should examine a section from each batch of paraffin with a polarizing microscope. A finer crystalline paraffin gives adequate support.

Warm paraffin shrinks as it cools and compresses the tissue in the block. Tissue that is harder than the paraffin withstands this pressure, but soft or spongy tissue may be under considerable strain. When sectioned, the tissue tends to expand to the shape and size it had before compression, and if confined by the paraffin around it, pleating or wrinkling results. Stowell reported that Parowax shrinks in volume 14.3% on cooling from 59° to 20° C.

Unless the hardness of the paraffin is adapted to the temperature at which the cutting is done and the nature of the tissue, good sections may not be expected, regardless of the excellence of the microtome and sectioning knife. When the material has been properly dehydrated, it is possible to re-embed it in another paraffin should the first not prove satisfactory. Poorly prepared tisue can rarely be salvaged. Several changes of the melted paraffin should be used to wash out the former paraffin.

Fisher Tissuemat: Fisher Tissuemat* is an embedding compound that will neither crumble nor crack while being cut on the microtome. It is well adapated for ribbon sections and is more convenient to use than paraffin cakes because it is supplied in granular form. It melts rapidly and obviates cutting large cakes. The granules are protected by an inner container of cellophane.

Histowax: Histowax† is an excellent substitute for paraffin, will not crumble or crack during the cutting process, and is well adapted for making ribbon sections.

CUTTING AND MOUNTING PARAFFIN-EMBEDDED SECTIONS

Cut out the tissue blocks embedded in the paraffin pool, in the paper boxes, by trimming away the paper with a scalpel or other sharp knife; then shape them into square blocks. Leave at least 1 mm. of paraffin around the blocks and keep the upper and lower surfaces parallel. Then place the blocks in pillboxes bearing their proper case numbers and store in a cool place or refrigerator until ready to cut.

When ready to cut sections, place the blocks in ice water.

Metal or wooden object holders are used to mount the paraffin block on the microtome. Warm the holder enough to melt the paraffin, heating it in a Bunsen flame for a few seconds. Press the paraffin block onto the holder; then pass a hot needle around the edges of the base of the block to aid in firmly attaching it to the holder. If the blocks are large, it may be necessary to run a hot needle under the center of the block to aid in attaching it.

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*Fisher Scientific Co., Pittsburgh, Pa.

†Matheson-Coleman & Bell Division, The Matheson Company, Inc., E. Rutherford, N. J.

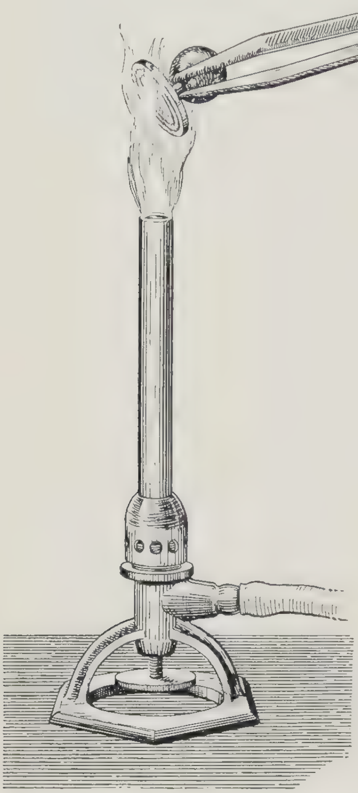

Fig. 535. Heating the paraffin microtome pivot.

When the block is firmly attached to the holder, quickly dip it into the ice water to cool and harden. When sectioning is completed, remove the remainder of the block from the holder, label it with an adhesive type label, and store in a refrigerator.

When the object holder and paraffin block have cooled, proceed with the cutting. Set the microtome knife at a 90° angle to the direction of the cut (no slice angle) and tilt it to as little clearance as will give good sections (2°-6°). Be sure the knife is sharp and shows no serrations.

Use the pencil type of camel's-hair brush to handle the ribbon (unless single sections are to be made). Sometimes it is necessary to hold the first few sections onto the knife with gentle pressure of the brush until the ribbon forms. Set the automatic lift on the mircotome for the desired thickness of the section and begin the cuttting process. Raise the end of the ribbon with a dissecting needle over the brush. Withdraw this from the knife (with the left hand) as the ribbon lengthens, turning the microtome wheel with the right hand. Speed of cutting varies with the tissue. Proceed until the ribbon lengthens to a point at which it is too long to handle. Place the ribbon lengths on smooth paper or in a shallow box until they are mounted on slides. Experience is required to cut serial sections without the loss of parts of the ribbon. Do not allow the brush to come in contact with the knife or it will be spoiled. Do not use a dissecting needle in place of the brush, since in case of accident of this type the knife will be damaged.

One great inconvenience of paraffin sectioning, particularly in winter or if the atmosphere is dry, is the flying of sections and their sticking to parts of the microtome and other nearby objects because of static electrical charge generated by the friction of cutting. Raise the humidity by boiling water in the room or burning a Bunsen burner near the microtome to alleviate this difficulty. Sometimes it is necessary to ground the microtome to a nearby water pipe, using a wire or a chain.

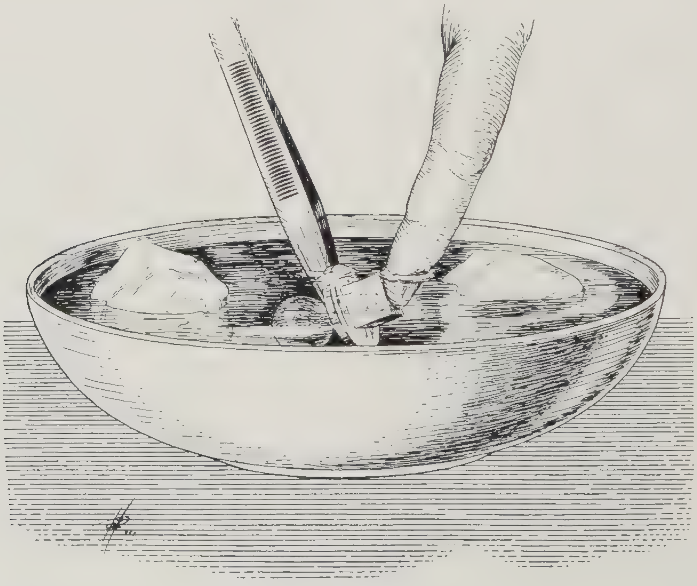

Fig. 536. Cooling the paraffin block after mounting it on the pivot.

After cutting the ribbon it must usually be flattened before the sections may be attached firmly and evenly to the slide.

Flattening the ribbon: Cut the ribbon into convenient lengths. Place on the slide containing albumin fixative. Add a few drops of water to the slide, and warm it gently until the sections expand to their full size.

Place the pieces of the ribbon onto warm water, allow to expand, and recover them by placing a slide containing a fixative under the tissues to pick them up.

Water used for "floating out" should be 5-7° cooler than the melting point of the paraffin. Commercial wetting agents may be added to the water if desired. Do not stretch the section with needles or by gravity by holding the slide perpendicular when draining off the excess water.

Cutting individual sections: Individual sections are cut in the same manner as above, but removed by means of a brush or teasing needle and spread on warm water. Routine sections are cut 7-10μ thick, but for research purposes they should vary between 3 and 5μ. Small foldovers and wrinkles can be flattened out by stretching the section with a teasing needle.

Tissue flotation bath: The tissue flotation bath with automatic temperature regulation is an excellent and convenient apparatus for floating and spreading of tissue sections embedded in paraffin, preparatory to mounting. Bath temperatures can be controlled to within ±1° C. in the range from room temperature to 60° C. It consists of a Pyrex glass tray, 10½ in. long by 6½ in. wide by 2 in. deep, and a low, insulated, Monel metal stand with flat copper top and built-in heating unit, thermoregulator, and pilot lamp. The top has raised edges, and a black oxidized finish, which aids in the in-

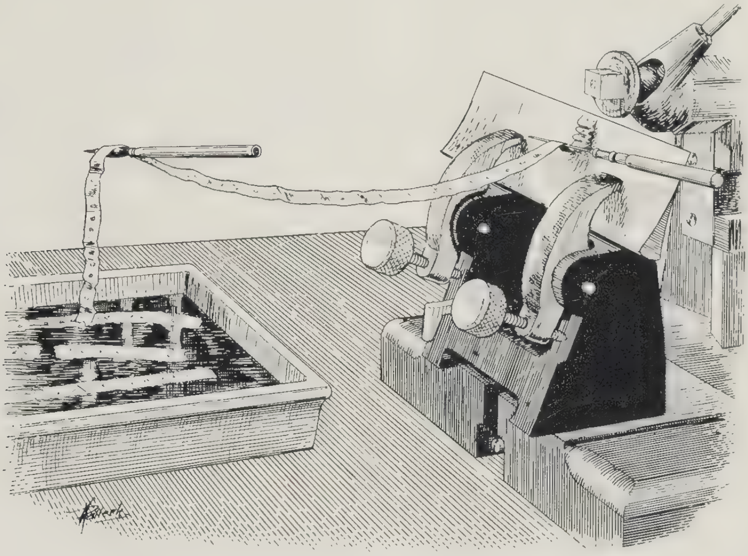

Fig. 537. Cutting paraffin sections and floating the ribbons on warm water

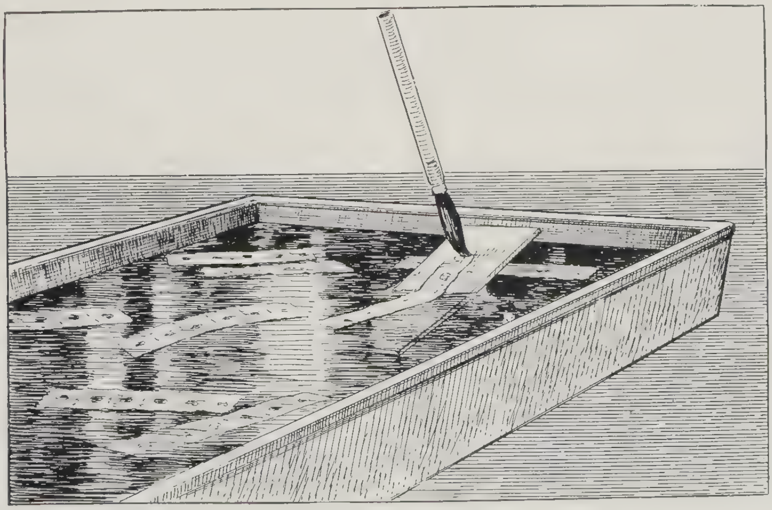

Fig. 538. Placing paraffin sections on a glass slide.

spection of translucent sections. The glass tray is readily removable for cleaning. The power consumption is 225 watts.

Affixing sections to slides: Have ready some clean slides and a diamond pencil for marking them. When mounting ribbons, cut to the desired lengths.

Rub the surface of a slide with a drop of egg albumin fixative.

Pick up the first strip of the ribbon on the slide and let the water drain off. Repeat the process for every strip. Mount single sections as directed on p. 1677.

Number each slide with its proper case number and place it in a slanting position in a wooden box holding 25 slides.

At times it is necessary to flatten out sections before subjecting them to the drying process. Hold the sections over a Bunsen burner at a temperature several degrees below the melting point of the paraffin, about 45°-50° C. Dry the sections by placing them in a paraffin oven at 55° C. for 3-5 hr. to coagulate the egg albumin fixative thereby causing them to adhere firmly to the slide.

If sections are to be stained in aqueous solutions for many hours, the drying must be prolonged or they will fall off the slide. Using thin celloidin as described on p. 1666 will prevent loss of sections.

When the sections are properly dried, stain by a standard staining method.

Faults in dehydration, clearing, and embedding, and the remedies

If the tissues are insufficiently dehydrated, they cause the clearing reagent to turn milky. To correct this, dehydration with absolute alcohol should be repeated.

If the tissues are insufficiently cleared, they become opaque and section cutting is impossible because of the presence of alcohol. Repeat the process of clearing, leaving the tissues in the clearing reagent for 2 hr.

The room temperature and the condition and the position of the knife and paraffin block markedly affect the cutting of good sections. The morning is preferable in warm weather, as the temperature is more favorable. It is essential to have a basin containing chipped ice ready to keep the paraffin blocks cool.

In cutting paraffin sections, sometimes the ribbons are curved instead of straight. This is caused by lack of parallelism of the surfaces of the blocks and may be remedied by unscrewing the block holder and trimming the block properly.

Sometimes sections are cut well individually but do not adhere to one another to form a ribbon. This is due to too cold a block. Warming the knife over the flame or in warm water corrects this condition.

Crumbling of the sections is caused either by too soft paraffin or too dull a knife. This may be prevented by cooling the block in ice water and sharpening the knife by stropping.

Irregular cutting of the sections, sometimes thick and sometimes thin, is caused by improper adjustment of the knife or block holder and may be corrected by tightening the screws of the block holder and the knife. If scratching of the section occurs as a result of nicks on the knife, the nicks should be removed by honing.

Difficulties in cutting paraffin sections

1. As block is being cut, there is a gritty feeling or a scratchy noise. Paraffin oven was too hot, there is dirt in the paraffin, or fixing agent may have crystallized and was not washed out with water.

2. Sections jam on the knife: knife is dull, or paraffin is too soft, or paraffin has accumulated on the edge of the knife. Clean with xylol.

3. Paraffin rolls: knife is dull, or sections are too thick.

4. Objects crumble and drop out of the paraffin: insufficient paraffin penetration before embedding.

5. Ridges on specimen: nicks in knife or paraffin accumulated on edge of knife.

6. White patches near tissue in paraffin: caused by xylol carried into paraffin. Reembed if near tissue. Cut off if near edge of block.

Difficulties in cutting ribbons of paraffin sections

1. Ribbons fail to form. (a) Room is too cold or paraffin is too hard. Use softer (lower melting point) paraffin; warm knife slightly by blowing the breath on it or immersing it in warm (not hot) water; or place a desk lamp so that the light and heat fall on the knife and block. (b) Tilt the knife less. (c) Cut thinner sections. (d) Knife may be too dull—resharpen. (e) Dip block into a softer paraffin and trim so that a thin layer remains on the upper and lower edges of the block. (f) Unroll the section and hold it lightly against the knife with a camel's-hair brush. If the first few sections can be held down, the ribbon will often form and follow.

2. Crooked ribbons. (a) When sections are wedge shaped, the sides of the block are not trimmed parallel. (b) Edge of block may not be parallel to knife edge. (c) Try another part of the knife—sometimes irregularities of the

knife edge cause crooked ribbons. (d) Or the paraffin at one side of the block may be softer than at the other side, especially if the material has been re-embedded in a paraffin of different hardness—re-embed the material and stir the melted paraffin.

3. Sections compressed, wrinkled and jammed together. (a) The knife may be too dull. (b) Or the room may be too warm; cool trimmed block and knife in very cold or ice water immediately before sectioning or re-embed in a harder paraffin. (c) Tilt the knife more; the knife tilt may be too slight so facet bevel rubs over block. (d) The knife edge may be gummed with paraffin; wipe with finger or cotton moistened with xylol. (e) Soak block in water from an hour or two to overnight before cutting. (f) If cutting is done too rapidly, thin tissues will wrinkle.

4. Sections crumble and specimen may tear out. (a) Material incompletely dehydrated or not properly cleared. (b) When soft and mushy, material incompletely infiltrated—reinfiltrate and embed. (Salvage rarely possible if material was incompletely dehydrated.) (c) Alcohol not completely removed by clearing fluid. (d) Object too long in paraffin bath or paraffin too hot. (e) Subject hard and brittle due to clearing fluid. Try toluene in place of xylol or a mixture of toluene and cedar oil. (f) Try celloidin embedding or a rubber or asphalt mixture with paraffin for fragile material. (g) Try dioxane method for dehydrating.

5. Split ribbon or lengthwise scratches in ribbon. (a) If there are nicks in knife, resharpen or use another knife. (b) Use less tilt of knife so it will cut rather than scrape. (c) Knife edge may be dirty. (d) Object may be too large for paraffin method—use celloidin. (e) Hard particles in block may cause scratching. If there is dirt in the paraffin, filter or decant melted paraffin. There may be crystals from killing fluid (mercuric chloride); washing was insufficient. If calcareous or silicious particles are present, decalcify or desilicify.

6. Knife rings on upstroke and sections are scratched. (a) Change knife tilt to greater or lesser degree—tilt must be sufficient to clear facet bevel but not enough to scrape instead of cut. (b) Material is too hard. Soak in water to soften; clearing may be at fault. (c) A thicker or wedge-shaped knife may prevent springing of the edge when cutting. (d) Material may be too tough for paraffin method—try celloidin.

7. Sections lifted from knife on upstroke. (a) Increase knife tilt. (b) Room too warm or paraffin too soft—try harder paraffin or cooler room; or cool the block. (c) Knife may be dull—resharpen.

8. Sections stick to knife. (a) Knife edge dirty. (b) Increase knife tilt. (c) Try a sharper knife.

9. Undulations in the surface of the section. (a) Tighten all screws on knife and block holders and see that knife holder is clamped fast to microtome base. (b) Lessen excessive knife tilt to prevent vibration.

10. Scratching noise during cutting. (a) Material may be too hard or small regions of material may be hard.

11. Sections fly and stick to parts of microtome or other nearby objects because of static electricity formed from the friction of cutting. This usually occurs only in winter when the air is very dry. (a) Increase humidity of room by boiling water in an open pan or burn a Bunsen burner in the room. (b) Ground microtome to a water pipe with a wire or a chain. (c) Ionize the air by an electrical method.

12. Sections vary in thickness or are skipped. (a) Knife not tilted enough to clear facet or bevel, or tilted too much, and tissue is compressed until the inevitable expansion gives a thick section. (b) Some of the clamping set screws on the block or knife holder are not tight or knife holder block is not clamped firmly. (c) Microtome worn through lack of lubrication or is not in adjustment. (d) Very large blocks or blocks with hard regions may spring knife edge while sectioning—soak block in water to soften, use other methods for softening the material, or embed in celloidin. The block will soak quicker if the paraffin is trimmed off one side to expose the tissue. Very little water is absorbed, but this process sometimes makes possible the cutting of hard or tough material.

Serial sections

To obtain serial sections by any of the 3 methods, it is only necessary to avoid losing any of the sections cut. The paraffin method is preferable to the others because by this method long ribbons are cut and placed on a sheet of paper in proper order. These can then be divided with a teasing needle into short series of any desired number of sections and fastened to numbered slides by means of egg albumin-glycerin fixative.

Vacuum embedding

Vacuum embedding or embedding under reduced pressure is used for certain purposes. A special type of apparatus is necessary, in which the embedding chamber is so arranged as to be airtight. The procedure consists in partially reducing the pressure during impregnation with paraffin. The necessary reduction in pressure is effected by a hand exhaust pump or by a water aspirator.

The advantages of the method are (1) the time required for embedding is considerably reduced, the clearing reagent being more rapidly replaced by the paraffin (this reduction in time is important in tissues that become unduly hard during the process of embedding) and (2) any air present in the tissue is extracted.

For the vacuum thermostat the embedding chamber is a circular brass well, closed at the upper end by a thick glass lying upon a rubber ring. The chamber is kept at the proper temperature by a water jacket, while a small tube connects it with the exhausting apparatus (vacuum pump or aspirator). There is also an arrangement in the shape of a small screw plug, fitted into the wall of the chamber for the admission of air. When the apparatus is used in conjunction with water aspirator, a glass bottle or a thick-walled flask of at least 500 ml. capacity should be placed between aspirator and thermostat. This prevents water from being sucked directly into the embedding chamber should the water supply to the aspirator be inadvertently turned off before opening the air valve. It is advantageous to attach a mercury manometer to the apparatus.

Procedure:

1. Place the cleared tissue in pure paraffin within the apparatus.
2. Reduce the pressure very slowly. A reduction to 400 or 500 mm. Hg is usually sufficient. The time required for impregnation varies from ¼ to ½ of that required for the same objects at atmospheric pressure.
3. Slowly admit air through the air valve until the pressure within the apparatus is in equilibrium with that of the atmosphere. For large objects repeat the process of exhaustion 2 or 3 times, changing the paraffin each time. To ensure success the exhaustion and the readmission of the air must be very gradual. Delicate air-containing tissues are damaged by too rapid a change.

The vacuum method is indicated for air-containing tissues, especially lung tissue; for large and dense objects, e.g., whole embryos or large pieces of brain; for tissues that become unduly hard if the embedding bath is prolonged, e.g., fibrous tissue and muscle; and for any work in which speed is important.

Technic for small pieces of tissue, skin, etc., using Bouin fixative

Fix tissue overnight in Bouin solution (p. 1641); 24 hr. is the maximum.

First day:

9:00 A.M.	Wash in 50% alcohol.
10:00 A.M.	Wash in 70% alcohol containing about 3 drops of a saturated solution of lithium carbonate.
12:00 noon	Place in 70% alcohol (may be left over the week end in this solution).
3:00 P.M.	Place in 80% alcohol.
5:00 P.M.	Place in 95% alcohol.

Second day:

9:00 A.M.	Place in absolute alcohol.
10:30 A.M.	Place in amyl acetate.
4:00 P.M.	Place in paraffin in a paraffin oven.
5:00 P.M.	Pour off paraffin and leave tissue outside of paraffin oven. Never leave tissue in oven overnight. Never re-use any paraffin.

Third day:

9:00 A.M.	Place in second paraffin.
12:00 noon	Place in third paraffin.
2:00 P.M.	Embed. Cut and stain with routine hematoxylin and eosin stain.

Amyl acetate can be used for clearing formalin-fixed tissues as well as those fixed in Bouin solution.

If tissue is fixed in formalin, first wash in water from 9 A.M. until 10:30 A.M.

Then place in 50% alcohol and then in 70% alcohol until 12 noon. The technic for Bouin-fixed tissue is followed from this point. Staining is with hematoxylin, eosinol, etc., p. 1691.

ROUTINE METHODS OF EMBEDDING AND CUTTING PARAFFIN SECTIONS

Alcohol dehydration method for paraffin embedding

Reagents:

1. Formalin, 10%. Described on p. 1641.
2. Alcohol, 70%.
   Dilute 70 ml. 95% ethyl alcohol or isopropanol to 95 ml. with distilled water. Wait for the bubbles to stop rising and then dilute again to 95 ml.
3. Alcohol, 80%.
   Dilute 80 ml. 95% ethyl alcohol or isopropanol to 95 ml. with distilled water, carrying out the same technic.
4. Alcohol, 100%.
   Absolute alcohol can be kept at 100% concentration by mixing it with pulverized copper sulfate that has been previously dehydrated. The copper sulfate falls to the bottom of the bottle, the 100% alcohol remaining above.
5. Xylol.
   Purchase chemically pure or U.S.P. xylol (xylene, water- and acid-free).
6. Paraffin. See pp. 1674 and 1675.

7. Mayer albumin fixative. Refer to p. 1652.

Procedure:

1. Fix tissue blocks in 10% formalin at least 2 hr. (After a month in formalin it is difficult to treat the section.)
2. Wash 1 hr. in tap water or a shorter time in running water to remove the formalin. The tissue may be kept in the same bottle throughout the dehydrating and clarifying processes.
3. Dehydrate in
   70% alcohol* 1 hr. (longer, if necessary; stopping point, 1 hr.)
   80% alcohol 1 hr. (longer, if necessary; stopping point, 1 hr.)
   95% alcohol 1 hr. (longer, if necessary; stopping point, 1 hr.)
   100% alcohol not more than 1 hr.
   These solutions may be used repeatedly until a sediment appears.
4. Place in xylol 1 hr. to clear. If not clear, immerse for a longer period. It is opaque until placed in xylol, when the edges become clear. If there are milky or chalky spots in the tissue, put it back in 95% alcohol, then absolute alcohol, and then xylol.
5. Place in xylol-paraffin (equal parts) all night with just enough heat to keep liquid. **Do not put in the paraffin oven.**
6. The following morning place in pure paraffin for 2-2½ hr. in an oven at 58°-60° C.
7. Transfer to pure paraffin again. Tissues may be left here a day if necessary, but 4-5 hr. is sufficient. The oven must not be hotter than 58°-60° C. or 2° above the melting point of the paraffin.
8. Embed or orient, using the paper box or "boat" (p. 1674) or other devices for the purpose.
9. Trim the blocks when hardened, attach to a wooden block or pivot (p. 1675), and label the back of the block. Retain the label throughout the process.
10. Cut the tissue according to directions on p. 1676 and float in warm water.
11. Mount with Mayer albumin fixative (p. 1652 for fixative and p. 1678 for affixing sections to the slide) on a glass slide.
12. Allow to stand in an upright position until dry.
13. Stain, mount, and label.

Acetone-benzine method for routine paraffin embedding (Krajian)—preferred method

1. Fix thin blocks of tissue in 10% formalin for 16-24 hr. In emergencies fix for 30 min. in a paraffin oven at 56° C.
2. Place in acetone for 1 hr. using at least 20 times as much acetone as tissue.
3. Place in a second acetone for 1 hr., using the same quantity of acetone.
4. Place in benzine for 30 min.
5. Place in second benzine for 30 min.
6. Place in paraffin No. 1 in the paraffin oven for 1 hr.
7. Place in paraffin No. 2 in the paraffin oven for 1 hr.
8. Embed in the usual manner.

This method is useful for surgical tissues.

Alcohol-chloroform method for routine paraffin embedding

1. Dehydrate tissues in 70% alcohol for 2-3 hr.
2. Dehydrate in 80% alcohol for 2-3 hr.
3. Dehydrate in 95% alcohol for 2-3 hr.
4. Dehydrate in 95% alcohol (second time) for 2-3 hr.
5. Dehydrate in absolute alcohol for 2-3 hr.
6. Place in chloroform for 2 hr.
7. Place in a paraffin oven at 55° C. in a mixture of equal parts of chloroform and melted paraffin for 2-3 hr.
8. Place in pure paraffin in a paraffin oven at 55° C. for 2-3 hr.
9. Embed in the usual manner.

This method is excellent for autopsy specimens.

Benzine method for routine paraffin embedding

1. Dehydrate tissues in 70% alcohol for 2-3 hr.
2. Dehydrate tissues in 80% alcohol for 2-3 hr.
3. Dehydrate tissues in 95% alcohol for 2-3 hr.
4. Dehydrate tissues in 95% alcohol for 2-3 hr.
5. Dehydrate tissues in absolute alcohol for 2-3 hr.
6. Place in benzine for 2-3 hr.
7. Place in benzine and melted paraffin, equal parts, in a paraffin oven at 55° C. for 2-3 hr. For the evaporation of benzine leave the corks out of the bottle; otherwise the fumes may cause an explosion.
8. Place in pure melted paraffin in the paraffin oven at 55° C. for 2-3 hr.
9. Place in second paraffin bath in paraffin oven at 55° C. for 2-3 hr.

Dioxane or isopropanol in the process of embedding tissues

Dioxane*: Using graded alcohols, as described above, in the preparation of tissues requires time and necessitates constant attention. One of the bugbears in paraffin work is over-hardening of the object, making it difficult or impossible to cut sections without tearing or cracking.

Dioxane (diethylene dioxide) has been suggested as a dehydrating agent, and it has proved remarkably efficient. It is miscible in all proportions with water and gives up water and methyl or ethyl alcohol to anhydrous calcium chloride; it is also miscible with melted paraffin. If the tissue is put into an

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*Isopropanol of appropriate strength may be substituted for ethyl alcohol in all dehydrating processes but **not** in the staining solution formulas.

*Union Carbide Chemicals Co., South Charleston, W. Va.

alcoholic solution of dioxane containing calcium chloride, the water is removed and the tissue is permeated with dioxane. Upon transfer of such a specimen to melted paraffin the dioxane is removed and the tissue is infiltrated with paraffin ready for sectioning when cool.

Isopropanol*: The use of isopropanol to replace graded alcohols, absolute alcohol, and xylol, except when reference is made to acid alcohol, is also recommended. It is economical, being less expensive than ethyl alcohol.

Method 1:

1. Cover the bottom of a vessel with calcium chloride and put a fine wire net over it, on which the objects are placed.
2. Cover the vessel to prevent evaporation of the dioxane and absorption of water from the atmosphere.
3. After fixation take the specimen from the fluid (formalin) and place it directly into the dioxane and leave it there for 3-24 hr.
4. Transfer to a paraffin oven at 55°-60° C. in a mixture of ⅓ dioxane and ⅔ paraffin, and then put it into pure paraffin.
5. Keep the pure paraffin in a dish with gauze a short distance above the bottom since the dioxane tends to sink through it.

Method 2:

1. Fix tissues in any desired fixative in the usual manner. Zenker-fixed tissues must be washed 16 hr. before being subjected to dehydration.
2. Dehydrate in 25% dioxane for 1 hr.
3. Dehydrate in 50% dioxane for 2 hr.
4. Dehydrate in 100% dioxane for 3 hr.
5. Place in paraffin at 56° C. for 30 min.
6. Place in paraffin at 56° C. for 1 hr.
7. Place in paraffin at 56° C. for 3 hr.
8. Embed in paper boxes.

Dioxane is **highly flammable**, and the vapor is quite toxic. **Excessive exposure to the vapor might cause serious consequences.**

Butyl alcohol in paraffin embedding

1. Fix tissues in 10% formalin for 24 hr. or longer.
2. Dehydrate in 80% ethyl alcohol for 3 hr.
3. Dehydrate in 90% ethyl alcohol for 20 hr.
4. Place in butyl alcohol No. 1 for 3 hr.
5. Place in butyl alcohol No. 2 for 3 hr.
6. Place in butyl alcohol No. 3 for 15 hr.
7. Place in paraffin No. 1 in a paraffin oven at 56° C. for 1 hr.
8. Place in paraffin No. 2 in a paraffin oven at 56° C. for 1 hr.
9. Place in paraffin No. 3 in a paraffin oven at 56° C. for 1 hr.
10. Embed in paraffin in paper boxes.

EMBEDDING TISSUE WITH A WATER-SOLUBLE WAX

Blank and McCarthy[20] developed a method for embedding tissues which is suitable for skin, liver, kidney, spleen, lung, testis, muscle, spinal cord, pancreas, adrenal, gastrointestinal tract, etc. It is especially applicable to delicate specimens such as skin with vesicles, eyes, embryonic membranes, etc. This method eliminates time-consuming, deleterious, dehydrating chemicals.

Embedding mixture:

Carbowax 4000	9 parts
Carbowax 1500	1 part

1. Melt these substances together at 55° C.

Solution for floating and affixing sections to slides (optional):

Potassium dichromate . .	0.2 gm.
Gelatin	0.2 gm.
Distilled water	1000 ml.

1. Boil in daylight for 5 min. cool, and filter. Ready for use.

Procedure:

1. Cut blocks of tissues, fix, and wash in the routine manner. As with other methods, the thinner the specimen the more rapid the impregnation with wax.
2. Immerse in the melted embedding medium and agitate occasionally. The minimum time for impregnation is 30 min. for a 1 mm. section, 80 min. for a 2 mm. section, and proportionately longer for thicker sections. Specimens have been left in melted Carbowax for periods up to 4 days with little perceptible distortion. The routine time should be about 3 hr.
3. After impregnation, transfer to a mold and add fresh melted wax to form a block.
4. Transfer the mold to a cool dry place, such as a refrigerator, and allow to harden for 5-10 min. Avoid contact with ice and water because of the solubility of the wax.
5. Attach the hardened block to a microtome specimen holder, and it is ready for sectioning. The block may be cut at 2-10μ with the formation of satisfactory ribbons. The knife and block should not be moistened or chilled because best results are obtained at room temperature.

Sections may be hydrated and affixed to the slide either by water or gelatin-dichromate solution. The latter keeps the sections firmly in place on the slide during subsequent staining procedures.

AUTOTECHNICON SYSTEM FOR PARAFFIN SECTIONS*

The Autotechnicon is a method especially designed for fixation, dehydration, and embedding of specimens by use of a special automatic apparatus. It is adaptable to any technic and, once set, adheres to that technic, eliminating human error. It requires no supervision since it operates automatically. The apparatus consists of a timing clock, reagent beakers of Pyrex glass, beaker platform, mastershift carriage, beaker covers, displacer rotor, receptacle basket, receptacles, washer, and paraffin bath. Figs. 539 and 540 show the equipment.

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*Union Carbide Chemicals Co., South Charleston, W. Va.

*We are indebted to the Technicon Co., Inc., Chauncey, N. Y., for permission to prepare the section on the Autotechnicon.

The Autotechnicon is especially recommended for overnight and week-end tissue routine. To use the instrument for staining, plug in a 1 hr. staining clock and replace the processing fluid beakers with others containing the staining fluids required by the prescribed technic.

Typical daily hospital routine for embedding paraffin sections with Autotechnicon system: At a convenient time, usually in the late afternoon, the pathologist receives the tissues that have been collected during the day. While preparing and examining the specimens, he dictates the gross aspects into a recording apparatus. It is convenient to have a permanent microphone set up at the cutting board, with a foot switch to start and stop the recorder so that both hands are free for processing. Such pieces should be no more than 4 mm. thick, regardless of the other dimension. As the pieces are cut they are placed in a tissue receptacle fitted with dividers that enable it to hold several specimens for simultaneous processing. During this procedure the receptacle bottoms should be stored on one tray, dividers on another, and tops on a third. The pathologist can then select the tissues, pick the proper dividers, place them in the receptacle, and finally snap on the cover in a continuous operation.

If the pieces are small, the receptacle may hold 3 pieces; if larger, 2; or if very large, only 1. Paper identification slips are placed in the receptacle along with the tissue. Slips should be no larger than 3 × 10 mm. so as not to impede penetration of the fluid. Notations made with an ordinary graphite pencil remain legible during the ensuing processing and immersion in paraffin. As each receptacle is filled it is placed in the receptacle basket, which is immersed in a beaker of fixative lying beside the cutting board. Receptacles should be placed vertically in the basket rather than horizontally, for better penetration and draining during processing.

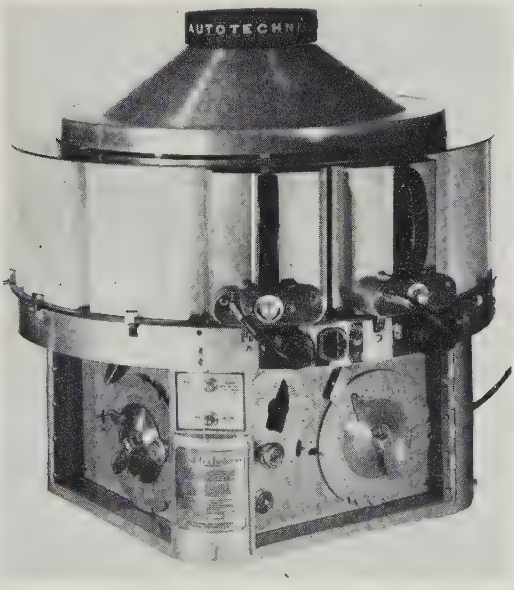

Fig. 539. Mono Autotechnicon. (Courtesy The Technicon Co., Inc., Chauncey, N. Y.)

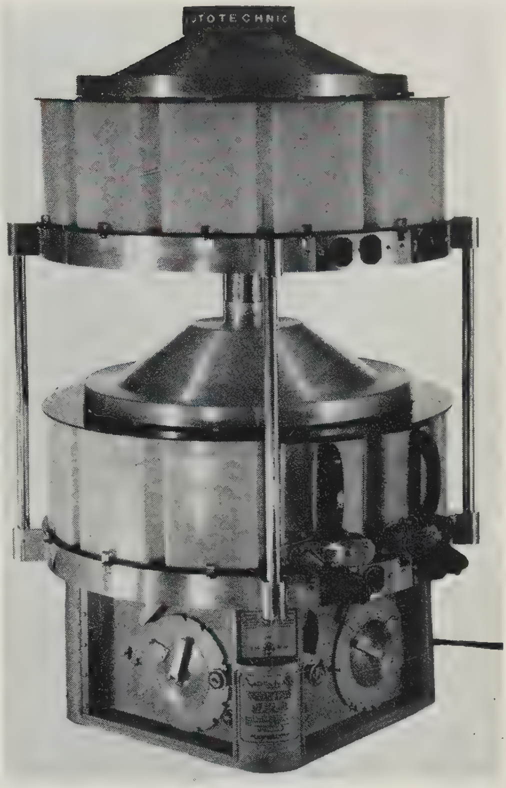

Fig. 540. Duo Autotechnicon. (Courtesy The Technicon Co., Inc., Chauncey, N. Y.)

When the receptacle basket has been filled, the displacer rotor is attached to it, and the whole assembly, including the beaker of fixative in which it has been lying, is mounted on the Autotechnicon. Then a check is made to see that no paraffin adheres to any of the beaker covers, since it might solidify and cause the beaker to tip and spill when the carriage rises.

The machine is now started and, from this point on, takes over. Precise time periods for each step in the desired procedure have already been cut into the timing dial.

Assume that the machine has been started at 5 P.M. In a typical routine, the tissues will remain in the fixative for 4½ hr., until 9:30 P.M. A stable solution, Technicon fixative, with all the properties of Zenker fluid but with none of its disadvantages, is recommended. It is chemically balanced to preserve the selectivity of the ensuing stain. Although it is a potassium dichromate formula, tissues fixed in it need not be washed since it produces no appreciable precipitate. It does not harden tissues, and they may remain in it for prolonged periods without harmful effects. Subsequent immersion in a

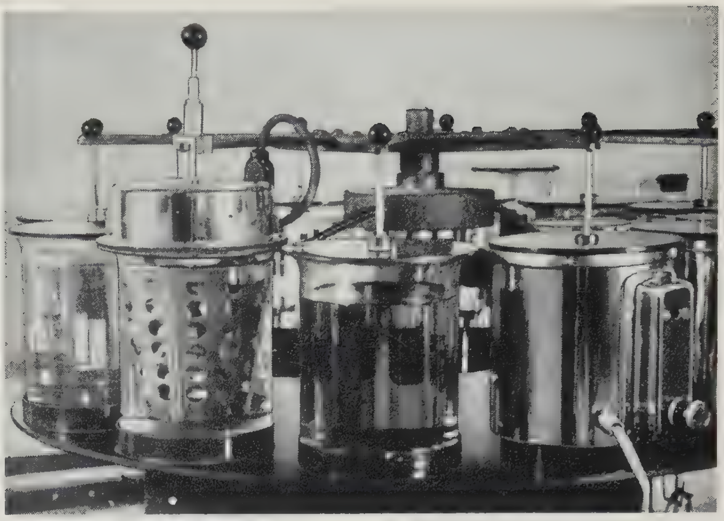

Fig. 541. Displacer rotor is attached to receptacle basket and the whole assembly mounted on Autotechnicon. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

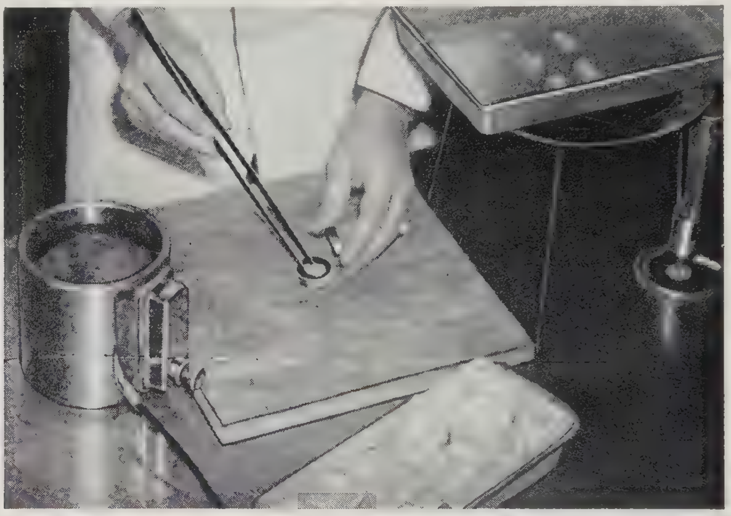

Fig. 542. Tissues are removed from their receptacles with forceps and placed in a flat pan of melted paraffin. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

beaker of water or the tissue washer for a period of 30 min. is recommended in order to wash off excess fixative and avoid contamination of the reagents that follow.

From 10 P.M. to 4 A.M. the tissues go through 6 changes of dehydrants, 1 hr. for each change. Technicon Dehydrant is neither hygroscopic nor highly volatile, does not take up atmospheric moisture, and is practically without loss from evaporation. It does not cause tissue shrinkage, nor does it harden. The tissues may be left for prolonged immersion without damage. Its physical properties materially lessen fire hazard.

From 4 to 6 A.M. tissues go through 2 stages of 1 hr. each of clearing. The Technicon clearing agent is an excellent medium for clearing. It manifests somewhat the same qualities as the dehyrant, i.e., is not volatile, hygroscopic, or markedly flammable and produces no shrinking, distortion, or hardening of the tissues. It is excellent for clearing fatty tissues. It removes all the dehydrant and at the same time conditions the tissues so that they will cut easily. It is highly soluble in paraffin, renders the tissues translucent, and increases their refractive index.

From 6 to 7 A.M. the cleared tissues enter and remain in the first paraffin bath. From 7 to 9 A.M. they undergo a second paraffin treatment. Although the first bath becomes contaminated, the second remains reasonably free of contaminants. Paraffin used in infiltrating the tissues should have a relatively low melting point, 54°-58° C., so that tissues are not subjected to too high a temperature for prolonged periods.

At 9 A.M., or whenever the working day starts, the paraffin bath is lifted with its contained load of

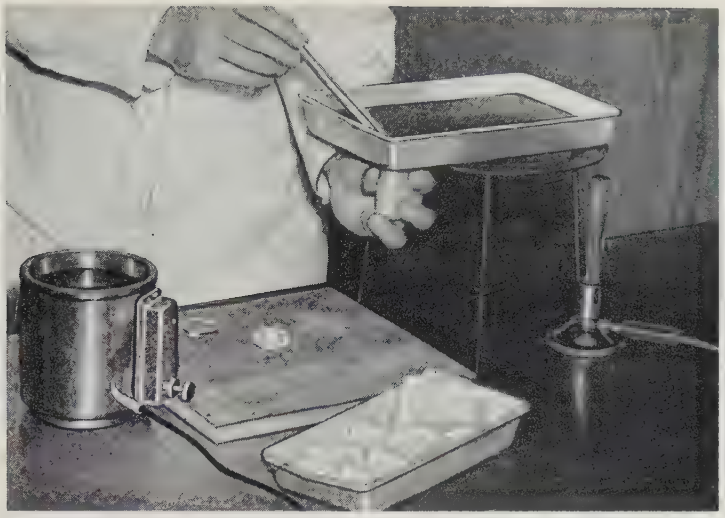

Fig. 543. Rubbing ice along bottom of pan causes the paraffin to solidify locally, anchoring tissues securely in place. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

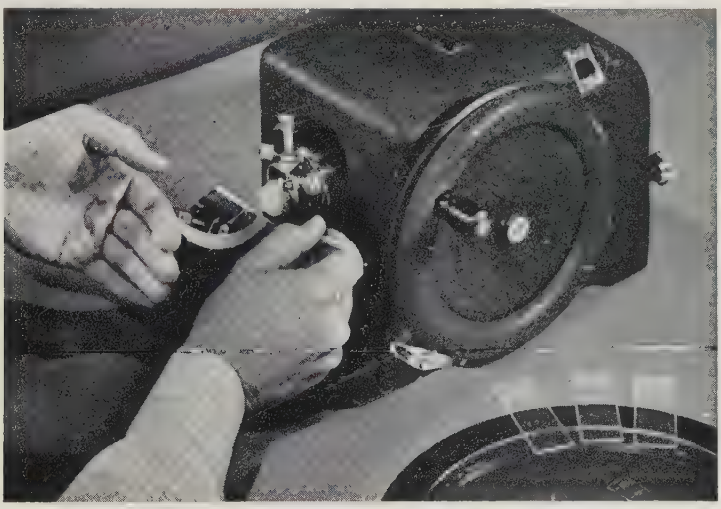

Fig. 544. Cut tissues are lifted carefully from microtome and spread face up on Technicon constant temperature water bath. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

tissues in the receptacle basket and carried to the embedding bench; then the bath is plugged into an electric outlet to maintain the paraffin at the desired fluidity.

Preparing the tissue blocks: Remove the tissue receptacles one by one from the paraffin and lay them on the table, bottom down. With the handle of the forceps strike the top lightly, which causes the clamping lugs to spring open with a snap that breaks loose any adhering paraffin.

Using the same forceps remove the tissues from the receptacle, each with its identifying label, and place in a flat pan of melted paraffin. For embedding purposes use a paraffin with a melting point of 60°-62° C.

Place a Bunsen burner or similar heating device beneath the pan to keep the paraffin fluid during embedding. Set the pan on a fairly high stand so that ice may be rubbed along the bottom. This causes the paraffin to solidify locally, anchoring the tissues securely in place. The pan should contain about ½ in. of paraffin, and the tissues should be embedded with the side to be cut facing down.

Before pouring in the paraffin, lightly coat the pan with glycerin so that the final paraffin slab can be easily removed when embedding is completed.

Place the tissues in straight rows so that neatly squared blocks can be cut when the paraffin slab is divided for sectioning. Cool the slab slowly to prevent crystal formation. It is best to let it stand at room temperature until the paraffin congeals. Once congealed, the paraffin slab should be cooled in water for hardening. Do not use too low a temperature since this will probably cause the paraffin to form crystals, making the tissues difficult to cut.

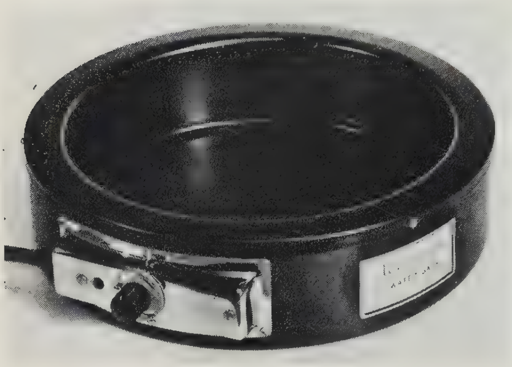

Fig. 545. Constant temperature water bath for tissue flotation. (Courtesy The Technicon Co., Inc., Chauncey, N. Y.)

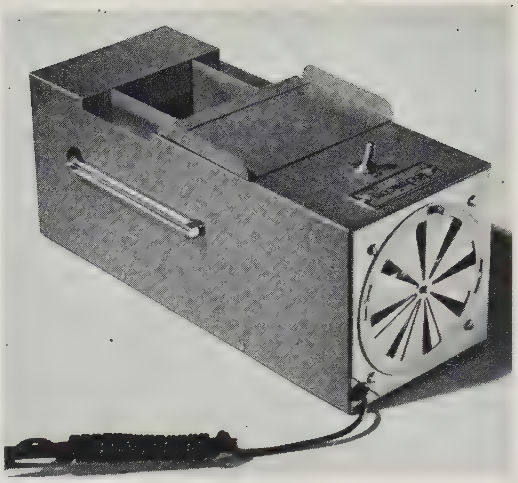

Fig. 546. Technicon microslide dryer. (Courtesy The Technicon Co., Inc., Chauncey, N. Y.)

When the hardened slab is freed from the pan, cut it into individual blocks, each containing a piece of tissue with its accompanying label.

From this point proceed to mount the block, cut the sections, affix them to slides, and stain in the usual manner.

Staining with the Autotechnicon: Insert the slides containing the tissue into a Technicon microslide dryer, where a current of warm, filtered air at 56° C. passes over them, completely drying the slides in about 7 min.

Take the dried slides, still in the rack, from the drier and mount the rack on the Autotechnicon for staining. Operation from this point is completely automatic. The tissues move into a beaker of xylol for 2 min. and then into 0.25% iodine dissolved in the dehydrant to remove any mercuric chloride crystals persisting in the tissue. They remain in the iodine for 2 min. and then go for 2 min. into a bath of Technicon dehydrant to remove any excess iodine. The next step is distilled water for 2 min. and then hematoxylin for 10 min., more or less, depending on the intensity of color required. The Technicon hematoxylin needs no subsequent decolorization. The next step is a rinse of distilled water for 4 min. then 0.01N lithium carbonate solution for 2 min., followed by distilled water for 2 min. Next, the tissues go for 5 min. into eosin solution. (The Technicon eosin solution will remain active under steady use for approximately 6 mo. No subsequent decolorization is required with this solution.)

The tissues pass into distilled water, then for 5 min. each in the dehydrant for 2 baths, and then into xylol. With the use of tongs lift the staining rack with its load of stained slides and place upside down in a beaker of xylol.

Remove the slides from the rack singly, clean, and mount with a cover glass. The slides, once labeled, are ready for microscopic examination.

Chapter 90

Celloidin sections*

ARAM A. KRAJIAN

Celloïdin methods are used for large tissues and organs and for hard or delicate materials. The celloidin is not removed from the tissues and holds the delicate structures permanently together.

The material to be cut is embedded in one of the commercial celloidins (cellulose nitrate), the slow burning kinds being preferred. The disadvantages of the method are that a longer embedding time is necessary, serial sections cannot be cut, so that each section has to be handled individually, and only dyes that do not stain celloidin may be used.

For materials not injured by moderate amounts of heat, the rapid process facilitates impregnation and preparation of the tissue. The slower, cold process takes more time and does not damage the tissue. It may not be possible to embed a hemisphere of a brain or a whole lung and harden it sufficiently for cutting in less than 6 mo.-1 yr.

The chief difficulty in celloidin sectioning arises from trying to cut improperly prepared material. Inadequately hardened blocks cannot be sectioned successfully. The blocks should be dense enough to cut at the required thickness. Unless the block is sufficiently hardened, the sections are uneven and distorted. If the block is too hard, irregularities are likely to occur. The surface of the block and the surface of the knife should be kept wet with 70% alcohol, and as soon as the sections are removed they should be placed in alcohol.

The sliding microtome is the instrument of choice for cutting celloidin material, using a slice angle of 10-40°.

Tilt the knife a little more than for cutting with paraffin, though the actual tilt will depend upon the hardness of the tissue. It is convenient to let large sections roll up on the knife. Then lift off of the knife and unroll into 70% alcohol. Or remove the sections from the knife and lay a piece of filter paper on top of the section. It usually adheres to the filter paper and then both may be placed in a Stender dish. If the filter papers are numbered, they may be kept in order. Or stamp a number on the margin of the section with a commercial numbering machine as each one is cut.

The knife for sectioning celloidin-embedded tissue must be very sharp. Any irregularities in the edge will leave marks on the section. Keep the section and materials for embedding free from dust. Dust particles catching on the edge of the knife spoil the section, particularly when very thin sections are required. Remove silicious or calcareous material before sectioning. Do not allow the surface to dry. If it is necessary to stop sectioning for even a short time, cover the block with absorbent cotton and saturate with alcohol or remove the block and place in 70% alcohol or other storage liquid.

The so-called dry process has been developed to replace the method which requires keeping the block and knife wet with alcohol. After the tissue is embedded and hardened, soak the block in an oil (cedar oil) which lubricates the block during cutting. As the sections are removed from the knife, place them in the same oil. The oil-soaked block may be cut on a rotary microtome, since no slicing cut is required. This method is exceptionally good for making sections of difficult organs, such as the entire mammalian eye.

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*We are indebted to Dr. O. W. Richards for this material from his pamphlet "The effective use and proper care of the microtome," American Optical Co., Scientific Instruments Division, Buffalo, N. Y.

Special knife holders are available so that the knife can be turned to give a slicing stroke and may be used for cutting small celloidin blocks on a rotary microtome. The position of the block and knife is such, however, that it is difficult to keep the surfaces properly lubricated with alcohol so that the rotary microtome is not very satisfactory for this type of sectioning. If much sectioning in celloidin must be done on a rotary microtome, it is advisable to use the dry method which does not require a slicing stroke.

Celloidin-embedded blocks are sometimes embedded in paraffin to hold them for sectioning. The size of the section that may be cut depends on the size of the microtome and the knife and the skill of the operator.

Celloidin sections are usually stained in Stender dishes or watch glasses. They are not affixed to the slides until ready for mounting. At times it is more convenient to stain them on the slide by the following method.

1. Transfer the sections from water to the slide and dry by blotting in fine filter paper.
2. Cover with absolute alcohol for ½ min. and again blot.
3. Dip in thin celloidin (p. 1666) and drain off the excess quickly. Blow the breath briskly over them and immerse in water.

Celloidin sections can be stained by almost all methods without removing the celloidin. When it is necessary to remove the celloidin, place the sections in oil of cloves or ether-alcohol mixture for 5-10 min. and then pass through absolute alcohol into 95% alcohol.

During the process of staining, most celloidin sections swell and shrink. To flatten them, treat in equal parts of absolute alcohol and oil of cloves for 2 or 3 min. before placing in xylol.

Difficulties encountered in handling celloidin-embedded material

1. The chief difficulty comes from trying to cut improperly prepared material. Adequate impregnation of a large organ, such as a hemisphere of a brain, may take a year. Improperly hardened blocks cannot be sectioned successfully. Pressure methods involving heat speed up the process and are available when warming will not injure the material.

2. Lengthwise scratches or splits in the section may be due to the following: (a) Nicks in the knife. Use a different part of the knife or resharpen. (b) Particles of hard material in the block. Dust or dirt in the celloidin stock solution—let stand and use only upper portion after the particles have settled or filter the stock solution. If calcareous or silicious deposits are present, decalcify or desilicify.

3. Specimen falls out of section, is mushy and soft. (a) Dehydration was incomplete. (b) Infiltration was incomplete—refilter, reembed, and harden. (c) Harden block, if too soft, in chloroform or a mixture of equal parts of 95% alcohol and glycerin.

4. Variation in thickness of sections. (a) Loose screws on knife or block holders—tighten all set screws. (b) Knife holder depressed or raised by the hand while sectioning—hold knife block so as not to move it vertically while cutting. (c) Knife not tilted enough to clear facet of cutting bevel. (d) Knife too dull. (e) Microtome worn and out of adjustment. (f) Material not hardened properly. (g) Slight drying of block between sections.

Celloidin embedding and sectioning methods

After they are properly fixed in any given fixative, tissues are subjected to 1 of the 3 celloidin-embedding processes described below.

Wet celloidin embedding:

1. Prepare tissue blocks not more than 2-3 mm. thick.
2. Harden and dehydrate in 80% alcohol for 24 hr.
3. Repeat in 95% alcohol for 24 hr.
4. Repeat in absolute alcohol for 24 hr.
5. Soak in a mixture of equal parts of absolute alcohol and ether for 24-27 hr.
6. Impregnate in medium celloidin (p. 1666) for 2-4 days or longer, depending on the thickness and consistency of the tissue.
7. Soak in thick celloidin (p. 1666) for 3-6 days.
8. Embed in a paper box or mount on vulcanized fiber.
9. Harden in a desiccator with chloroform vapor. To harden the celloidin lift the cover of the desiccator jar once or twice a day for a moment to allow evaporation and thickening of the celloidin. When the celloidin attains the right consistency, add several drops of chloroform to the bottom of the desiccator. The vapor hardens the celloidin in several hours for small objects or several days for large ones. When the celloidin is hardened, place the blocks in 80% alcohol.
10. To mount, trim the block to proper size, dip the base in thick celloidin, press it firmly on vulcanized fiber, and treat with chloroform vapor as described above.
11. Keep the block in 80% alcohol until it is sectioned.

Celloidin is a purified form of colloidon or nitrocellulose. For histologic work it is supplied in small quantities (ounce) in brown bottles.*

Dry celloidin embedding:

1. Follow the wet celloidin embedding process and mount the tissue blocks on vulcanized fiber.
2. Expose them to the air until the celloidin mass attains the right consistency.
3. Then instead of hardening in chloroform, place them in **Gilson mixture,** made of equal parts of chloroform and cedar oil. Add cedar oil at intervals of 1 hr. for small blocks and 10 hr. or longer for larger blocks, until the mixture is about 90% cedar oil. By this time the celloidin becomes quite transparent.
4. Then expose the blocks to the air until dry.
5. Keep the blocks in stoppered bottles and cut dry.

Celloidin-paraffin embedding: The following technic enables one to obtain serial sections by embedding celloidin blocks in paraffin mass.

1. Embed blocks in celloidin by the dry process.
2. Trim the celloidin blocks and transfer them from cedar oil into melted paraffin in an oven at 55° C. for about 2 hr.
3. Place the blocks in paper boxes containing fresh paraffin and harden by placing on a wet towel.
4. For cutting, follow the paraffin sectioning method.

Advantages and disadvantages of celloidin method

Advantages: For large tissues, long period of immersion in celloidin does not affect the consistency. Such objects treated by the paraffin method become very hard since prolonged infiltration is necessary to ensure complete penetration.

For hard objects, such as eye and bone tissue, celloidin provides better support than paraffin and does not crumble in sectioning.

Disadvantages: The disadvantages are (1) very thin sections cannot be obtained, (2) embedding process is very slow, and (3) serial sections are difficult to prepare.

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*Parlodion (Mallinckrodt) is recommended.

Chapter 91

Tissue staining methods

ARAM A. KRAJIAN

The use of dyes in histologic microscopy began about 1850. Carmine, indigo, iodine, fuchsin, aniline red, aniline violet, and other dyes were used in simple solutions. Hematoxylin was first introduced by Waldeyer[22] to stain axis cylinders in an aqueous solution. In 1865 Boehmer[23] combined hematoxylin with alum and thereby made it a valuable histologic stain, opening the way for more combination stains. The differential stains were introduced around 1890, with the use of various chemical reagents. Progress continued, and many new staining methods of considerable importance were introduced. Mallory[24] developed his famous aniline blue stain for connective tissue, Heidenhain,[25] developed his iron hematoxylin stain for protozoologic work, Levaditi[26] discovered a stain for *Treponema pallidum* in tissue, and Cajal[27] revolutionized the subject of neuropathology by his investigations and discovery of various staining methods for nerve fibers. Other investigators and authors have developed important methods for identifying such elements as glycogen, amyloid, fat, etc. in tissue.

The purpose of staining is to recognize different tissue elements by their color reactions. Because the physical chemistry of the cell and tissue elements is not well understood, it is not clear why certain tissues have an affinity for one dye and not for another.

Staining methods may be classified as (1) routine staining, (2) special or differential staining methods, and (3) vital staining.

ROUTINE AND SPECIAL STAINING

A routine method is one which stains various types of protoplasm generally, with little differentiation except between nucleus and cytoplasm. It demonstrates the cells and the general cellular arrangement of the tissue. Special staining methods are those of more limited scope, which demonstrate or differentiate special features of the tissue, such as the minute intracellular or intercellular structure, foreign materials, organisms, etc.

VITAL STAINING

Vital staining is employed to stain living cells by injecting the staining solution into any part of the animal body. It causes a specific coloration of the cytoplasm of certain cells, chiefly those of the reticuloendothelial system.

In employing vital stains a sterile syringe and needle are used. The staining solution to be injected should be free from particles or clumps which might cause emboli in the capillary vessels.

The vital dyes are usually injected intravenously, intraperitoneally, or subcutaneously. Smaller doses are needed for intravenous administrations, but large doses are necessary for subcutaneous and intraperitoneal injections.

Technic:

Lithium carmine:

1. Prepare a concentrated suspension of carmine, about 5 gm./100 ml., in cold saturated lithium carbonate. Filter and sterilize.
2. Inject 5-10 ml. intravenously into rabbits.

India ink (Higgins):

1. Dilute the India ink with an equal volume of sterile distilled water in a sterile test tube. Warm to body temperature before injecting.
2. The average intravenous dose is about 5 ml. for rabbits, repeated daily for 3-4 days, and then every 3 days thereafter.

Combination of vital stains: If desired, combine 2 or more types of vital stains; for example, one may inject India ink intravenously, trypan blue intraperitoneally, and lithium carmine intrapleurally. Some cells will ingest one dye, others may take up all the dyes.

SUPRAVITAL STAINING

Supravital stains are used to stain living cells immediately after removal from the living body.

Place thin slices of tissue in small staining dishes and add enough staining solution to cover the tissue. Several dyes are in general use.

Neutral red: This is a basic dye used for observing cell granules and vacuoles of phagocytic cells. It is employed on tissue slices in a concentration of 1:10,000 to 1:100,000.

Another method is to exsanguinate the animal and inject a 1:15,000 solution of dye in physiologic saline.

Janus green: This is regarded as a specific stain for mitochondria. Stain thin slices of tissue in 1:10,000 Janus green B in physiologic saline. Specific mitochondria stain may be given with a weaker solution, 1:500,000. Janus green B, 1:10,000 in physiologic saline, can be injected into animals previously bled to the point of exsanguination.

Trypan blue: For use, suspend 1 gm. of the dye in 100 ml. sterile distilled water and use immediately. It is dangerous to allow the suspension to stand more than 1 hr. because it is likely to become toxic.

Isamine blue and *Niagara blue* give similar results and are used in the same manner as is trypan blue.

ROUTINE STAINING OF SECTIONS

The method of staining frozen sections is given on pp. 1666 and 1668.

Routine hematoxylin-eosin staining of paraffin sections

Method 1—Recommended procedure[28]

This method is an adaptation of the staining technic devised by Krajian[29] for staining frozen sections. It gives excellent results. With this method sections are seldom lost during the staining process

1. Prepare paraffin sections in the usual manner. Cut them as thin as possible and mount on a glass slide without fixative. If a fixative is considered necessary, use Mayer albumin fixative. Allow the tissues to dry on the slide. Carry out the process from here in staining jars. (Fig. 528.)
2. Place slides in xylol No. 1 in a Coplin jar for 5 min.
3. Transfer to xylol No. 2 in a staining jar for another 5 min. to ensure removal of paraffin from sections. If necessary, use a third xylol bath.
4. Place slides in anhydrous isopropanol or absolute alcohol to remove the xylol. This usually requires 2 to 5 min.
5. Blot twice with several thicknesses of Whatman No. 1 filter paper.
6. Immediately dip twice in thin celloidin (p. 1666). Keep the slide perpendicular while dipping and dip quickly but not suddenly. Do not turn the slide while dipping in and out. Blow gently over the slide while holding it in a perpendicular position. Continue the gentle blowing until the celloidin is dry. Wipe the celloidin off the back of the slide. The slide is now ready to be stained, following the method for routine staining of frozen sections (pp. 1666 and 1668).

Method 2—Routine procedure

1. Flood the slide containing the cut section, previously affixed to the slide with Mayer albumin fixative, with pure xylol from a dropping bottle, placing sections on staining dishes for 5 min. Repeat the process.
2. Pour off the second xylol and flood the slide with absolute alcohol or anhydrous isopropanol twice, 5 min. each.
3. If the tissue is fixed in Zenker fluid or other fluid containing mercuric chloride, treat first in tincture of iodine 5 min. to remove the mercury precipitate, followed by **sodium thiosulfate (hypo) solution** until the natural color of the section reappears.

Sodium thiosulfate	0.75	gm.
Alcohol, 95%	10	ml.
Water	90	ml.

4. Wash in tap water.
5. Stain in Harris hematoxylin (p. 1658) 7-10 min.
6. Wash in a beaker of water until blue.
7. Destain in 1% acid alcohol until the reddish color of hematoxylin ceases to come off.
8. Wash in a large basin of tap water.
9. Blue the sections by dipping in 2% ammonia water (2 ml. ammonium hydroxide in 98 ml. tap water).
10. Rinse in tap water.
11. Stain in 1% aqueous eosin 5-10 min. or follow eosinol technic after dehydrating in isopropanol (p. 1668). When eosinol is used, omit the subsequent dehydrating steps.
12. Wash in 2 changes of tap water.
13. Dehydrate thoroughly in 2 changes of absolute or butyl alcohol or anhydrous isopropanol, 2 min. each.
14. Place in 2 changes of carbolxylol for 5 min.
15. Clear in pure xylol for 5 min.
16. Clear in a second pure xylol for 5 min. or longer.
17. Mount in gum damar.

Method 3—Routine procedure

Reagents:

1. Alcohols. The various strengths are described on p. 1680.
2. Hematoxylins. Harris, Delafield, Boehmer,

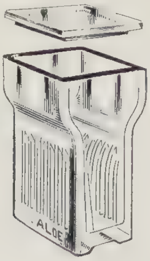

Fig. 547. Wide top staining jar. (Courtesy Aloe Scientific, St. Louis, Mo.)

Ehrlich, or Mallory phosphotungstic acid hematoxylin may be used.

3. Acid alcohol. 1% hydrochloric acid in 70% ethyl alcohol.
4. Alkaline alcohol.
    (a) Dissolve 2 gm. sodium bicarbonate in 100 ml. 70% ethyl alcohol
    or
    (b) Add 1 ml. concentrated ammonium hydroxide to enough 70% alcohol to make 100 ml. total.
5. Alcohol eosin, 0.5%.

Procedure: Prepare paraffin sections and mount on slides in the usual manner.

1. Put all solutions in Coplin jars or large staining jars (Fig. 547) and arrange the jars in the proper sequence. When transferring slides from one solution to another, catch the excess solution on a cloth as it drains off and dry the back of the slide with a towel. **Never let the section dry during the staining process.**
2. Immerse in xylol for 5 min. to dissolve the paraffin.
3. Place in absolute alcohol for 1 min.
    95% alcohol for 1 min.
    80% alcohol for 1 min.
    70% alcohol for 1 min.
    50% alcohol for 1 min.
    Do not make changes too rapidly or the section may fall off the slide.
4. Place in tap water for 1 min.
5. Stain in hematoxylin for 5 min. or longer. If the tissue has not stained the correct shade of purplish blue, replace the slide in the hematoxylin.
6. Rinse in tap water until no more color comes off.
7. Place in 50% alcohol. Then wash, just dipping the slide in and out.
8. Decolorize in acid alcohol. Dip the slide in the jar; then hold it above until the tissue is salmon pink. **Do not decolorize too long.**
9. Place in alkaline alcohol until blue.
10. Look at the tissue under a microscope to ascertain whether the nuclei are sufficiently blue. If not, replace the slide in acid alcohol, then 50% alcohol, then water, then hematoxylin, and then continue on from this point.
11. Place in 80% alcohol for 1 min.
12. Place in 95% alcohol for 1 min.
13. Dip in 0.5% alcoholic eosin Y about ½ min. or longer.
14. Wash in 95% alcohol but not too long since the eosin decolorizes.
15. Dehydrate in 100% alcohol but not too long; otherwise the eosin may disappear.
16. Finally place in xylol until clear, usually about 3-10 or 15 min. Look at the tissue under a microscope. If it is not red enough, pass the slide through the alcohols back to the eosin; if not blue enough, pass the slide back through the process to the hematoxylin.
17. Place 1-2 drops of gum damar over the tissue and mount with a cover glass without admitting any air bubbles.
18. Label.

Difficulties encountered in staining

If objects do not remain on the slide:

1. Slide may have been dirty or oily.

2. Albumin fixative is too old. (If too old, it loses its clear color. Or it may have an odor. Make it in 1 oz. quantities. This will last 2 or 3 mo.)

3. Slides might have been carried through the first alcohol baths too fast.

4. Dehydration of the tissue through alcohol may have been too rapid. (If tissue is too small, use 60% alcohol after the 50%.)

5. Paraffin section not thoroughly spread on slide when mounted.

If objects do not stain:

1. Stain is at fault. Hematoxylin must ripen. Do not use it too soon after preparation.

2. Stain has deteriorated.

3. Paraffin had not been thoroughly removed from the slide. Leave longer in xylol. The slide can be run back through the solutions to the first xylol.

4. Early fixation in alcohols before paraffin embedding was not correct; nothing can be done to remedy this.

5. Fixing agent not thoroughly washed out of tissue before immersing in alcohols.

If section is not clear under microscope:

1. Xylol should be replenished.
2. There was water in the absolute alcohol.
3. Moisture on the cover slip.
4. Too much egg albumin on slide.

5. All the acid after acid alcohol decolorization was not removed.

6. A film from the alkaline alcohol was carried along.

To remedy:

1. Put in Coplin jar of xylol to dissolve gum damar. Run back through the processes to the jar where the fault lay and use fresh solutions. Then restain.

2. If the tissue is thoroughly adherent to the slide, it can be taken back several times without danger.

Miscellaneous pointers

1. When mounting tissues on slides, make several at once.

2. Thick sections come off the slide.

3. Occasionally a tissue cannot be cut less than 10μ thick.

4. If the tissue is likely to come off (a) put slide in xylol to dissolve paraffin (b) dip in solution of ¾ gm. celloidin in 100 ml. ether-alcohol (equal parts). This places a slight film over the slide and does not interfere with the penetration of stain (except carmine).

5. After cover glass is on, if gum damar creeps out, rub off with xylol cloth.

6. Keep knives sharp.

7. If tissue is not embedded well, melt the paraffin in oven and re-embed it.

Eosin-methylene blue stain

Zenker and mercuric chloride-fixed tissue sections give the best results.

1. Deparaffinize sections by 2 applications of xylol, 2 min. each.
2. Remove xylol with 2 applications of absolute alcohol, 2 min. each.
3. Wash thoroughly in tap water.
4. Place the section in a jar containing 5% eosin Y and leave it in a paraffin oven for 30 min. at 52° C.
5. Rinse rapidly in tap water and immediately place in Borrel methylene blue; then leave it in a paraffin oven 30 min. at 52° C.
6. Wash in tap water. The section should be blue-black.
7. Differentiate in **colophonium-alcohol** (0.5 ml. colophonium in 100 ml. alcohol), agitating the slide constantly and examining every few seconds under a microscope.
8. Dehydrate rapidly in absolute alcohol.
9. Clear in xylol 5 min. and mount in gum damar.

Cell nuclei stain blue; cytoplasm, collagen fibers, and muscle stain pink; and erythrocytes and eosinophils stain bright pink.

Routine staining of celloidin sections

1. Stain loose sections in a Stender dish containing Harris hematoxylin for 2-5 min.
2. Wash in tap water until blue.
3. Destain in acid alcohol until the reddish color of hematoxylin ceases to come off.
4. Dip in ammonia water until blue.
5. Stain in 1% aqueous eosin 5-10 min. (or in eosinol 10-20 sec., after dehydrating in isopropanol).

Omit the next 3 steps if eosinol is used.

6. Wash in 2 changes of tap water.
7. Place in graded alcohols, 70%, 80%, and 90%, for a few minutes each.
8. Place in 90% alcohol for a few minutes.
9. Place in the following mixture not more than 5 min.: xylol, 1 part; chloroform, 1 part; absolute alcohol, 1 part.
10. Transfer to a dish of pure beechwood creosote. If wrinkled, the sections will immediately flatten out. Do not leave in the creosote more than 10 min. since celloidin is slightly soluble in creosote and alcohol.
11. Transfer the sections to glass slides.
12. Blot in filter paper.
13. Pour pure xylol on the slide, drain it off, and repeat the process once or twice. Do this as rapidly as possible since prolonged treatment with xylol will cause the sections to crease.
14. Blot rapidly with filter paper.
15. Mount in gum damar or Permount.

Chapter 92

Special or differential staining methods

ARAM A. KRAJIAN

STAINING COLLAGEN FIBRILS AND RETICULUM OF CONNECTIVE TISSUE

Mallory aniline blue stain, modified

Mallory aniline blue stain is not absolutely differential because in addition to collagen fibrils and reticulum it also stains hyalin, fibrin, fibroglia fibrils, smooth and striated muscle fibers, and amyloid.

1. Fix tissues in Zenker fluid.
2. Embed in celloidin or paraffin.
3. Cut thin sections.
4. Flood the slides with xylol for 5 min. Repeat.
5. Then flood slides with absolute alcohol or anhydrous isopropanol for 3 min. Repeat.
6. Rinse in water and place in tincture of iodine for 5 min.
7. Rinse in alcohol; then water.
8. Bleach with 5% sodium thiosulfate for 5 min. Rinse thoroughly in water.
9. Place in 1% acid fuchsin 5-10 min.
10. Rinse quickly in tap water.
11. Place in 1% aqueous phosphomolybdic acid solution for 1 min.
12. Rinse in water.
13. Stain for 5 min. in modified Mallory aniline blue (p. 1660).
14. Rinse in tap water and blot.
15. Dehydrate thoroughly with absolute alcohol or anhydrous isopropanol.
16. Clear in xylol for 5 min. Repeat in fresh xylol.
17. Mount in gum damar.

For celloidin sections use 95% alcohol and clear in xylol by the blotting method.

The collagen fibrils, reticulum, connective tissue, amyloid, mucus, and hyaline stain blue; nuclei, cytoplasm, fibroglia fibrils, axis cylinders, neuroglia fibers, and fibrin stain red; red blood corpuscles and myelin sheaths stain yellow; and elastic fibers stain pale pink or yellow.

If it is desired to bring out the collagen fibrils as sharply as possible, omit the staining with acid fuchsin. Then the nuclei and cytoplasm stain yellow, and the blue fibrilla and reticulum stand out more prominently.

This is an excellent and colorful method to demonstrate connective tissue fibers.

Azocarmine stain—Heidenhain modification of Mallory aniline blue

1. Fix tissue in Zenker solution 16 hr. Wash overnight. Pass through graded alcohols:
    - 80% alcohol overnight
    - 95% alcohol the next day
    - absolute alcohol overnight
2. Place in xylol for 2 hr. or more until tissue is clear.
3. Place in an oven at 56° C. for 30 min. Then change the paraffin.
4. Leave in oven for 3-4 hr. Embed.
5. Cut and mount sections and dry in an oven at 45° C. for 3-7 days. Pass slides through:
    - xylol for 5 min.
    - absolute alcohol for 3 min.
    - 95% alcohol for 3 min.
    - 80% alcohol for 3 min.
    - distilled water for 3 min.

NOTE: **Deformalinization**, if necessary, should be done at this stage, as follows:

Place sections in ammonia water (40 drops of ammonium hydroxide to 100 ml. distilled water). Leave in this solution for 7 hr.

(a) Wash in tap water for 1 hr.
(b) Place in Helly or Zenker fluid overnight.
(c) Wash in tap water for 1 hr.

This step is used only if tissues have been fixed in formalin.

6. Remove crystals with Lugol solution for 1 min. and then 2% sodium hyposulfite for 2 min. Wash in tap water for 5 min.
7. Stain sections in 1% acidified azocarmine (p. 1660) in an oven at 56° C. for 45-60 min. Allow to cool 5-10 min. at room temperature.
8. Wash in distilled water.

9. Differentiate in **aniline alcohol** 1-3 min.

Aniline oil	1 ml.
Alcohol, 95%	100 ml.

Nuclei stain red and cytoplasm stains pale pink (control under a microscope).

10. Remove aniline with alcohol acidified with 1% acetic acid for ½ min. If section is too red, put back in aniline-alcohol solution for a few seconds.
11. Place in phosphotungstic acid, 5%, for 2-3 hr.
12. Wash quickly in distilled water
13. Stain in Mallory aniline blue-orange G-acetic acid solution (p. 1660) for 3-6 hr.
14. Wash in distilled water.
15. Differentiate in absolute alcohol, controlling under the microscope.
16. Clear in xylol.
17. Mount in gum damar.

Amyloid, connective tissue, and mucous colloid take deep blue stain; nuclei are red.

This is a valuable stain to show minute details of connective tissue.

Krajian aniline blue stain for connective tissue and hyalin applicable to formalin-fixed frozen and paraffin sections

Frozen sections

Mordant:

Saturated aqueous solution of picric acid	25 ml.
Alcohol, 95%	25 ml.
Acetone	50 ml.
Thorium nitrate	4 gm.
Cadmium nitrate	5 gm.

1. Dissolve the thorium nitrate and cadmium nitrate in the picric acid solution; then add the alcohol and acetone.

Procedure:

1. Fix tissues in 10% neutral formalin as usual. In emergencies, fix in hot formalin in a paraffin oven at 56° C. for 15 min.
2. Cut thin frozen sections.
3. Place in the mordant for 5 min.
4. Rinse the tissue in tap water and transfer the sections to a glass slide.
5. Dehydrate with anhydrous isopropanol or absolute alcohol for 15 sec.
6. Blot and dip once in thin celloidin (p. 1666).
7. Dip in tap water and apply azocarmine fuchsin stain (p. 1660) for 5 min.
8. Rinse rapidly in tap water and treat with 10% aqueous phosphotungstic acid solution for 3 min.
9. Rinse in tap water and stain in Mallory aniline blue-orange G-acetic acid solution, p. 1660, for 5-8 min., using 2 vol. aniline blue solution and 1 part 10% aqueous citric acid.
10. Wash rapidly in tap water and control under a microscope. If a deeper shade of blue is desired, continue treatment with aniline blue until desired result is obtained.
11. Dehydrate rapidly with 3 applications of anhydrous isopropanol or absolute alcohol and follow with butyl alcohol for 2 min.
12. Clear in 2 changes of xylol, 1 min. each.
13. Mount in gum damar.

Collagen fibers stain deep blue; reticulum, light blue; hyalin, bright red; nuclei, red; erythrocytes, golden yellow; elastic fibers, dark red.

All the solutions should be kept in tightly stoppered bottles. They can all be used at least a dozen times, except aniline blue-citric acid combination, which should be discarded after it is used.

Paraffin sections

1. Fix tissues in 10% formalin in the usual manner and cut thin paraffin sections.
2. Mount on slides as usual.
3. Deparaffinize with 2 applications of xylol, 2 min. each, followed by 3 applications of anhydrous isopropanol or absolute ethyl alcohol.
4. Wash in tap water and place in the Krajian mordant (p. 1695) for 10 min.
5. Rinse in tap water and apply azocarmine-fuchsin (p. 1660) for 10 min., using 1 part stain with equal volume of 2% aqueous acid fuchsin solution.
6. Rinse in tap water and treat with 10% aqueous phosphotungstic acid solution for 5 min.
7. Rinse in tap water and stain in Mallory aniline blue-orange G-acetic acid solution (p. 1660) 8-12 min., using 2 vol. stain with 1 vol. 10% citric acid.
8. Wash rapidly in tap water and control under a microscope. If a deeper shade of blue is desired, continue staining with aniline blue until desired result is obtained.
9. Dehydrate rapidly with 3 applications of anhydrous isopropanol or absolute alcohol and follow with butyl alcohol 2 min.
10. Clear in 2 changes of xylol, 1 min. each, and mount in gum damar.

Van Gieson stain for connective tissue

The van Gieson stain is a selective method of demonstrating collagen fibers of connective tissue.

1. Stain frozen, paraffin, or celloidin sections rather deeply with alum hematoxylin, preferably the Harris formula.
2. Wash in water until blue.
3. Stain in van Gieson solution 5 min. (p. 1663).
4. Transfer directly to 95% alcohol, followed by absolute alcohol.
5. Clear in xylol and mount in gum damar or balsam.
6. For celloidin sections, clear in oil of origanum after 95% alcohol.

Cell nuclei stain dark brown; collagen fibers, bright red; muscle, erythrocytes, and other tissues, a transparent yellow.

Krajian modification of van Gieson stain

1. Prepare frozen, paraffin, or celloidin sections.
2. Stain for 5 min. in Krajian iron hematoxylin stain (p. 1658).
3. Wash in tap water.

4. Destain in 1% acid alcohol in 70% alcohol until no more color comes out.
5. Rinse in water.
6. Stain in van Gieson solution (p. 1663) for 5-10 min.
7. Rinse rapidly in water.
8. Blot dry.
9. Differentiate with equal parts of oil origanum and absolute alcohol or isopropanol for 5 min., changing the solution twice.
10. Clear in xylol and mount in gum damar.

The elastic tissue staining solution keeps well for long periods in well-stoppered bottles. It acts as a mordant in the van Gieson stain; it stains elastic tissue fibers, myelin sheath, and bacteria.

Cajal trichrome stain for connective tissue—Ramon Castreviejo modification

1. Fix tissue in 10% formalin.
2. Prepare frozen, paraffin, or celloidin sections.
3. Stain in acetic fuchsin formalin 2-5 min.:

Ziehl-Neelsen carbolfuchsin	15 drops
Glacial acetic acid	2 drops
Formaldehyde, 40%	2 drops

4. Wash in water.
5. Stain in the following solution 5-10 min.:

Indigo carmine	0.25 gm.
Saturated aqueous picric acid	100 ml.
Glacial acetic acid	5 ml.

6. Wash rapidly in water.
7. Dehydrate in 95% alcohol, followed by absolute alcohol.
8. Clear in carbolxylol, followed by xylol.
9. Mount in gum damar.

Nuclei stain deep red violet; cartilage, mucin, and mast cells, very intense bluish violet; cytoplasm, clear green or yellowish green; connective tissue, intense blue; and muscle fibers, clear green. This is a valuable connective tissue stain.

Perdrau-Bielschowsky method for reticulum

1. Fix tissue in 10% formalin.
2. Cut frozen sections 15-20μ thick.
3. Leave in distilled water 24 hr.
4. Treat sections for 10 min. in 0.25% potassium permanganate.
5. Wash and place in the following:

Oxalic acid	1 gm.
Potassium sulfite	1 gm.
Distilled water	200 ml.

6. Decolorize until white.
7. Wash thoroughly in several changes of distilled water overnight.
8. Place in 2% silver nitrate solution in the dark for 1-2 days.
9. Wash in distilled water for not more than 5 min.
10. Treat sections 40-60 min. in **Bielschowsky ammonium silver solution:**

Silver nitrate, 10%	5 ml.
Sodium hydroxide, 40%	5 drops
Distilled water	40 ml.

    Add strong ammonia drop by drop until precipitate is almost completely dissolved.
11. Wash quickly in distilled water.
12. Reduce in 20% formalin for 30 min.
13. Wash in distilled water. Tone in 0.2% yellow gold chloride solution.
14. Wash in distilled water. Fix in 5% aqueous sodium hyposulfite.
15. Wash, dehydrate, clear, and mount.

This is an excellent but slow method of demonstrating reticulum fibers.

Krajian rapid frozen section method for staining reticulum fibers

Ammoniacal silver solution: Place 20 ml. of 10% silver nitrate in a Stender dish. Add strong ammonium hydroxide drop by drop until the precipitate that is formed is almost completely dissolved.

Procedure:

1. Cut frozen sections 7-10μ thick.
2. Rinse in distilled water.
3. Place in the following potassium permanganate-ammonium hydroxide solution for 5 min.:

Potassium permanganate, 0.3% aqueous	15 ml.
Strong ammonium hydroxide	5 ml.

4. Rinse in distilled water for a few seconds.
5. Decolorize in 2% oxalic acid until all the brown color has disappeared (sections should not be left in this solution longer than necessary). Each section should be treated separately.
6. Wash in 2 changes of distilled water.
7. Place in warm ammoniacal silver solution, previously heated to 60° C. for 15 min., in a paraffin oven.
8. Wash in 2 changes of distilled water.
9. Reduce in warm 20% formalin for 2 min. (heated to 55° C.).
10. Rinse in distilled water.
11. Treat in 1:300 gold chloride solution for 2 min.
12. Rinse in distilled water.
13. Treat with 5% sodium thiosulfate solution for 2 min.
14. Wash and transfer to a glass slide.
15. When well drained off, mount in warm glycerin jelly.

This is a rapid and colorful method.

Foot method for staining reticulum

Silver ammonium carbonate solution:

1. Add 10 ml. saturated solution of lithium carbonate to 10 ml. 10% aqueous silver nitrate.
2. Allow the resulting heavy precipitate of silver carbonate to settle, and pour off the supernatant fluid.
3. Wash the precipitate several times in 25-50 ml. distilled water, allowing it to settle each time before decanting the supernatant fluid.
4. Add 25 ml. of fresh distilled water.
5. Dissolve the washed precipitate in strong ammonia water, adding it drop by drop while

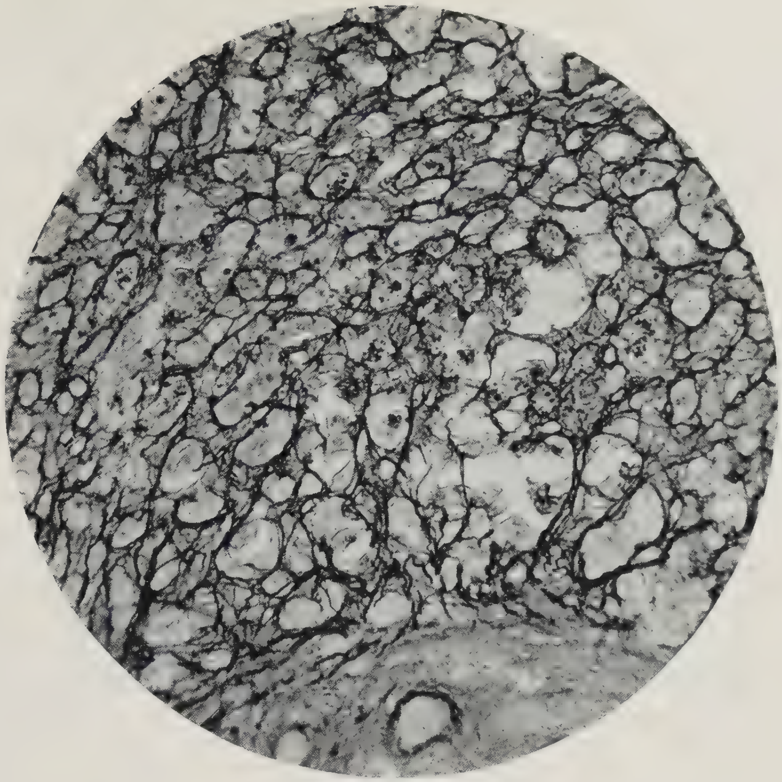

Fig. 548. Liver sections stained by Krajian rapid reticulum stain. Fibers of reticulum stain jet black. Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

shaking the container continuously. The precipitate turns grayish brown as the ammonia is added. About 8-15 drops of ammonium hydroxide are needed, depending on the concentration.

6. Dilute the entire solution to 100 ml. with distilled water and heat to about 50° C.

Procedure:

1. Fix tissues in Zenker solution for 24 hr.
2. Wash under running tap water for 12-24 hr.
3. Dehydrate and embed in paraffin in the usual manner.
4. Cut thin sections, 5-7μ thick, affix to slides, and dry thoroughly in a paraffin oven for 12 hr. or longer.
5. Dissolve the paraffin in 2 changes of xylol.
6. Wash in alcohol, followed by tincture of iodine for 5 min.
7. Wash in alcohol and bleach in 5% sodium thiosulfate.
8. Place in 0.25% potassium permanganate 5 min.
9. Place in 5% oxalic acid for 10 min.; then wash in tap water and then in distilled water.
10. Treat with silver ammonium carbonate solution, made fresh each time, by immersing the slides in the silver ammonium carbonate solution in an incubator at 37° C. for 10-15 min.
11. Wash quickly in distilled water and place the sections in 20% neutral formalin for 2 min., until they become dark brown.
12. Wash in tap water and transfer sections to 1:500 gold chloride solution for 2 min.
13. Wash in water and fix in 5% sodium thiosulfate for a few minutes.
14. Wash in water. If desired, use a counterstain.
15. Dehydrate the sections, clear in xylol, and mount in gum damar.

This is a rapid and useful method to demonstrate reticulum in paraffin sections.

Tumor reticulum*

The cells of the body are bound together by a stroma or network of connective tissue fibers of which 3 varieties are universally recognized: elastin, collagen, and reticulin. Collagen and reticulin appear to be closely related physically and chemically. Evidence is accumulating which may indicate that reticulin is a distinct substance formed by a precipitation-enzyme reaction in the secretions of certain cells such as reticulocytes, histiocytes, monocytes, lymphocytes, fibroblasts, smooth and striated muscle cells, and vascular endothelium. Reticulin is apparently a precollagen since typical collagenous fibers may be formed by the impregnation of reticulin fibers with collagen.

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*Much of the information regarding tumor reticulum is from Morhardt, R. P., and Krajian, A. A.: Atlas of tumor reticulum, Los Angeles, 1947, Mac Printing Co. Permission of authors and publisher has been granted to abstract this material.

Reticulum was first observed by Kupffer in 1876 during his histologic investigation of the liver. There is an increase in the sarcoma group of neoplasms rather than in the carcinomas. The reticulum stain as developed by Morhardt and Krajian impregnates epithelial cells, whereas those neoplasms of connective tissue origin exhibit fibers only. These authors are convinced that any undifferentiated cellular tumor that is without obvious characteristics is incompletely examined unless a reticulum stain is performed. They believe that carcinoma is never productive of an increase in reticulum and that sarcomas provoke an increase in reticulum; therefore, the use of reticulum stain offers a means of differentiating between sarcomas and carcinoma.

Morhardt and Krajian give a partial list of tumors, based on the amount of reticulum found, as follows:

Increased reticulum

Testicular seminoma
Pleural mesothelioma
Periphery of glioblastoma
Spinobasilar chordoma
Periphery of astrocytoma
Plasma cell myeloma
Retroperitoneal sarcoma, neurogenic
Giant cell tumor, osteoclastoma
Neurofibrosarcoma
Testicular lymphosarcoma
Periphery of metastatic melanoblastoma in brain
Leiomyosarcoma
Leiomyoblastoma
Reticulum cell sarcoma
Osteosarcoma
Lymphosarcoma
Monocytic leukemia (liver)
Plasma cell leukemia (liver)
Wilms' tumor (sarcomatous component)
Malignant lymphoma

Normal reticulum

Carcinoma of lung, primary
Fibroadenoma of breast
Neuroma
Uterine adenomyosis
Hemangioma
Lipoma
Neurilemoma
Dysgerminoma (ovarian seminoma)
Carcinoma of thyroid gland
Adamantinoma
Carotid body tumor

Diminished reticulum
(Displacement of normal tissue)

Hepatoma
Carcinoma of ovary
Carcinoma of testis
Neuroepithelioma
Glioblastoma, central portion
Glioma, central portion
Chorioepithelioma
Carcinoma, adrenal, metastatic
Medulloblastoma (center area)
Malignant melanoma, lymph node
Adrenal cortical adenoma
Ewing's tumor, lymph node
Carcinoma, pituitary, metastatic
Cystic disease of breast
Carcinoid in liver
Carcinoma of stomach

Staining tumor reticulum: A rapid and reliable method for the demonstration of reticulin fibers in frozen sections follows:

1. Fix blocks in 10% formalin.
2. Cut frozen section 7-10μ thick.
3. Place in a warm 10% ammonium hydroxide

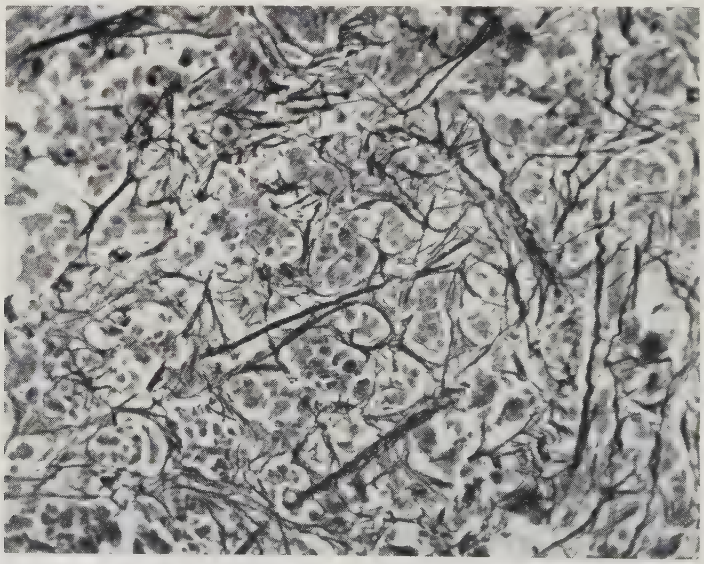


Fig. 549. Carcinoma of stomach. Note staining of carcinoma cells and absence of reticulum. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

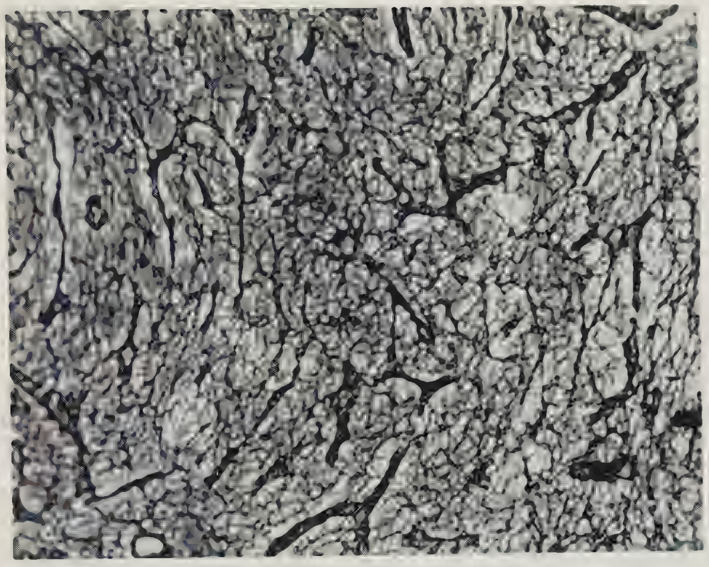


Fig. 550. Lymphosarcoma, showing great abundance of reticulum and absence of cellular staining. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

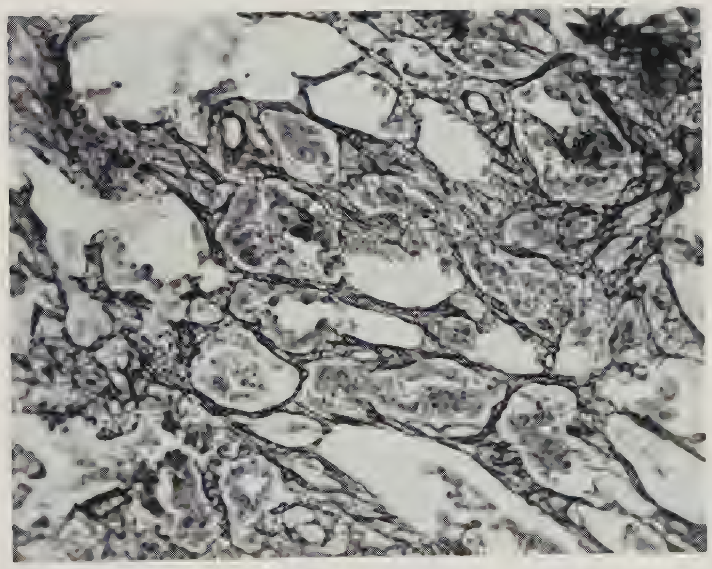


Fig. 551. Carcinoma of lung. Note staining of cells and absence of reticulum. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

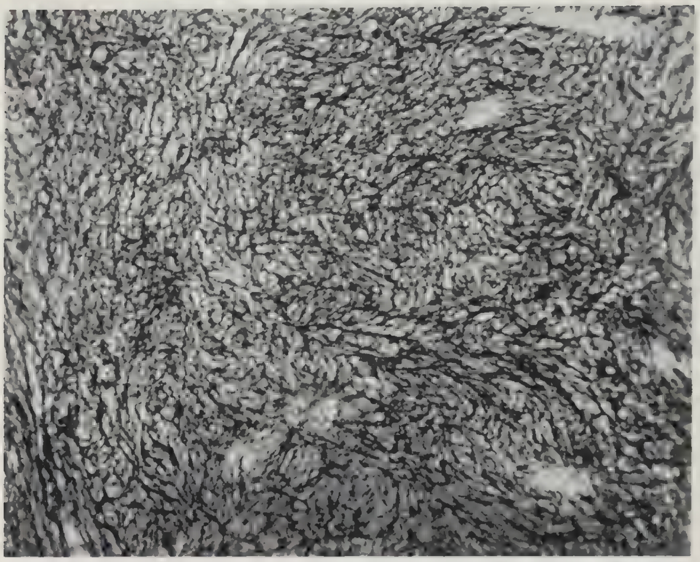


Fig. 552. Leiomyosarcoma showing great abundance of reticulum and absence of cellular staining. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

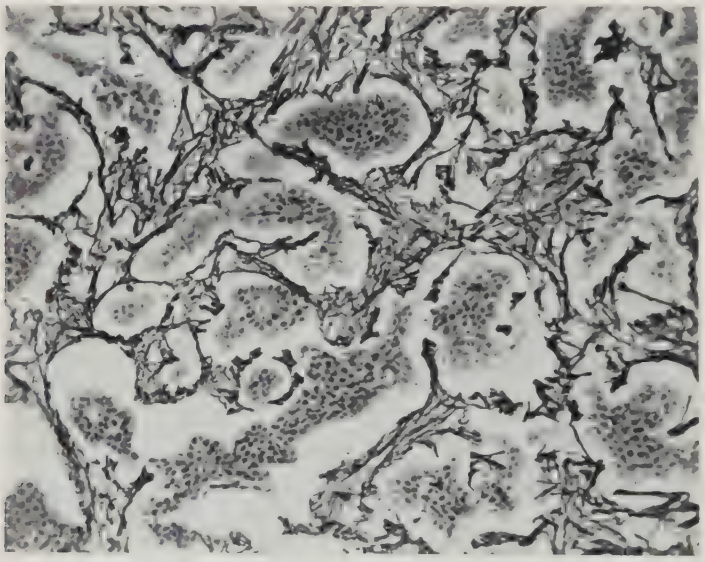


Fig. 553. Carcinoid of liver. Note deep stain of carcinoid cells and complete absence of reticulum. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

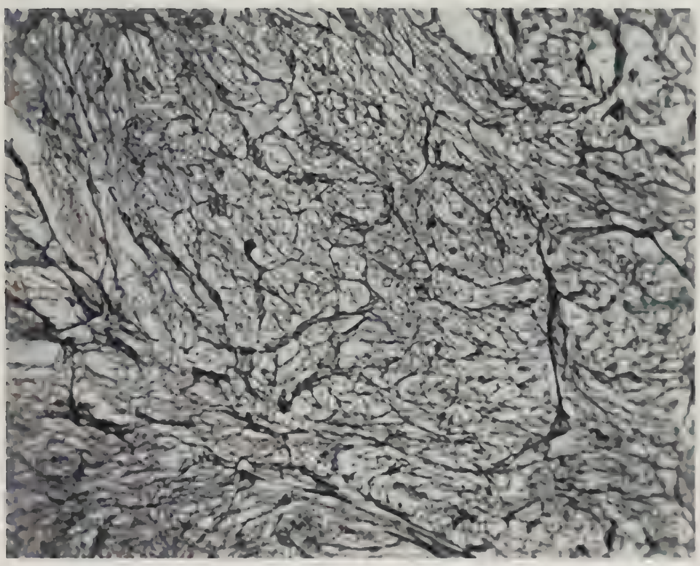


Fig. 554. Retroperitoneal sarcoma, showing great abundance of reticulum and absence of cellular staining. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

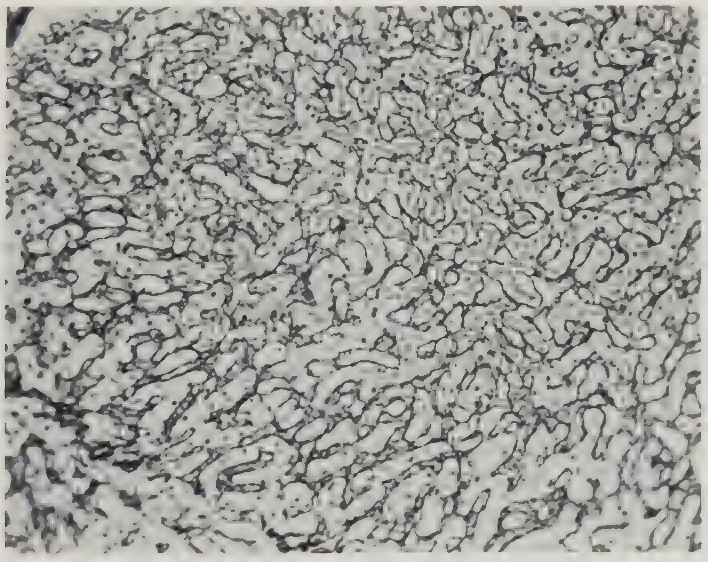


Fig. 555. Liver section showing normal reticulum. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

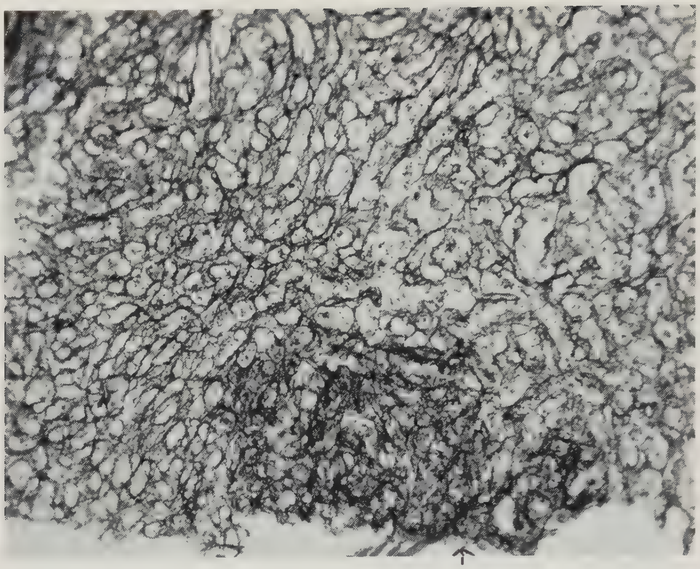


Fig. 556. Leukemia of liver, showing abnormal amount of reticulum (arrow). (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

solution in a paraffin oven at 56° C. for 10 min.

4. Wash rapidly in distilled water
5. Place sections in 0.25% potassium permanganate for 10 min.
6. Wash in distilled water and decolorize in 3% oxalic acid until most of the brown color is removed.
7. Wash in distilled water.
8. Place in a warm ammoniacal silver solution (p. 1696) and put in a paraffin oven for 40 min. at 56° C.
9. Wash in distilled water and place in warm 20% formalin for 3 min. (for reducing).
10. Wash in distilled water and place in 0.5% gold chloride solution for 3 min.
11. Wash in distilled water and place in 5% sodium thiosulfate solution for 5 min.
12. Transfer to a glass slide and dehydrate with 2 applications of anhydrous isopropanol or absolute ethyl alcohol, blot twice, and dip in thin celloidin, blow over the surface to dry section, and blot again.
13. Dehydrate twice with anhydrous isopropanol or absolute ethyl alcohol and apply creosote-xylol mixture for 3 min. (creosote, 1 part; xylol, 2 parts).
14. Clear in xylol for 3 min. and mount in Permount or gum damar.

Numerous methods have been devised for demonstrating reticulum and collagen fibers, but unfortunately most of the methods require paraffin embedding. The paraffin method not only delays the results but may in some way obscure some of the reticulin fibers which might be demonstrated if the frozen technic were used.

ELASTIC FIBER STAINS

Weigert elastic fiber stain

1. Fix tissues in formalin or alcohol. Zenker-fixed tissues stain slowly, and there is greater tendency to diffuse coloring of collagen fibrils. Embed tissues in paraffin or celloidin or cut frozen sections from formaldehyde-fixed tissues.
2. Stain sections for 20 min.-1 hr. with Weigert elastic tissue stain (p. 1661).
3. Wash in 95% alcohol.
4. Blot with filter paper and add xylol quickly.
5. Repeat the blotting, followed by xylol, 2 or 3 times, until the section is cleared.
6. Mount in gum damar.

The elastic fibers appear dark blue, almost black, on a clear background. The nuclei can be stained red with carmine before or after staining of the fibers. Carmine stain is hard to obtain after Zenker fixation. A light nuclear stain with some alum hematoxylin after the fibers have been colored is preferable. This is a useful and permanent stain.

Krajian method for staining elastic fibers[30]

This is a rapid method for elastic tissue, connective tissue, fibrin, and amyloid, employing Congo red.

1. Fix tissues in 10% formalin for 24 hr. or longer.
2. Cut frozen sections about 10μ thick.
3. Place in 2% aluminum chloride solution for 5 min.
4. Wash thoroughly in tap water.

5. Place in Krajian Congo red (p. 1662) for 10 min.
6. Wash quickly in tap water.
7. Transfer to 1% aqueous potassium iodide solution for 10 sec., rotating the section in the solution.
8. Wash thoroughly in tap water.
9. Place sections in Krajian aniline blue for elastic fibers (p. 1660) for 5-10 min.
10. Wash thoroughly in tap water in a large basin. Float sections onto slides and blot with filter paper.
11. Dehydrate in absolute alcohol for 2 min., changing the alcohol twice using the dropping bottle method.
12. Clear for 2 min. in oil of origanum.
13. Clear in pure xylol.
14. Mount in gum damar.

This method gives a colorful, well-differentiated preparation that keeps well when protected from direct exposure to light.

Elastic fibers appear bright red; fibrin and connective tissue, dark blue. Red blood cells are colored orange yellow.

Sections embedded in paraffin may be used satisfactorily, but they require longer staining periods. The staining technic is the same as that for frozen sections, except that it requires 30 min. staining in Congo red solution and 30 min. in the aniline blue solution.

If nuclear staining is desired in addition to differentiation of elastic tissue, stain sections in Harris hematoxylin for 2 min. after washing in tap water following the Congo red step. Wash, dehydrate, and clear in oil of origanum and xylol.

MacCallum modification of Verhoeff elastic tissue stain

1. Fix tissues in 10% formalin for 24 hr. or longer.
2. Cut frozen or paraffin sections.
3. Stain in MacCallum hematoxylin (p. 1658) for 30-60 min.
4. Wash in tap water.
5. Differentiate for a few seconds in 1% ferric chloride solution.
6. Wash in water.
7. Dehydrate completely in absolute alcohol.
8. Clear in xylol.
9. Mount in gum damar.

Elastic fibers assume a black color.

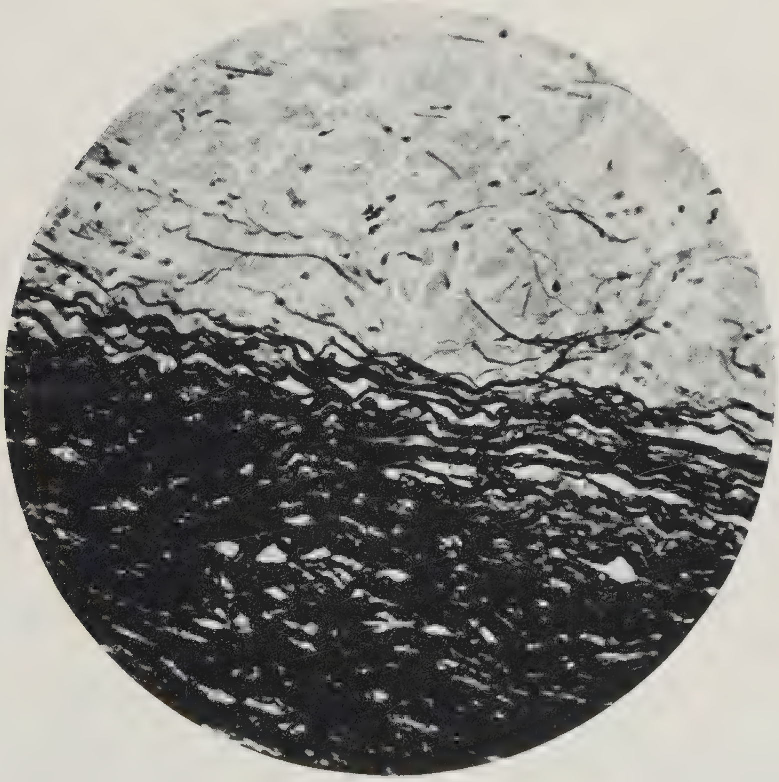


Fig. 557. Section of aorta from a case of syphilitic aortitis, stained with Krajian elastic tissue stain. Elastic fibers stain bright red; degenerated area remains unstained. Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

Krajian modification of MacCallum elastic tissue stain

1. Fix tissues in 10% formalin for 24 hr. or longer. In emergency, fix in paraffin oven at 56° C. for 15-30 min.
2. Cut frozen or paraffin sections in the usual manner.
3. Stain frozen sections 3-5 min. and paraffin sections 7-10 min. in the Krajian modification of MacCallum hematoxylin (p. 1658).
4. Wash in tap water.
5. Differentiate for a few seconds in 1% acid alcohol until the background begins to clear. Examine under the low power of a microscope.
6. Wash in tap water.
7. Treat with 2% aqueous zinc sulfate for 2 min.
8. Wash in water.
9. Dehydrate completely in absolute alcohol or isopropanol.
10. Blot and clear in xylol.
11. Mount in gum damar.

Treatment with van Gieson solution for a few seconds following washing after hematoxylin gives a combination of elastic and connective tissue stains. Prolonging the treatment in van Gieson solution will affect the elastic fibers. For this reason sections should be examined under a microscope for desired resuls.

FIBRIN STAINS

In fresh specimens fibrin swells and dissolves rapidly on treatment with 1% acetic acid.

Weigert fibrin stain

The Weigert fibrin stain is a modified Gram-Weigert method. It is by no means specific for fibrin, for it sometimes stains hyalin and coarse muscle fibers also.

1. Fix tissues in alcohol or formalin.
2. Stain sections on slides in 1% aqueous eosin solution or lithium carmine for 5 min.
3. Wash in water and stain in Sterling gentian violet 10 min. for paraffin sections and 3 min. for frozen sections.
4. Wash with physiologic saline.
5. Place in Gram's iodine solution for 3 min.
6. Wash with water.
7. Blot and destain in aniline oil-xylol mixture (equal parts) until the section is free from excess violet color.
8. Clear in 2 changes of pure xylol, blotting between changes.
9. Mount in gum damar.

The fibrin, gram-positive bacteria, and fungi are stained blue. The nuclei take a red stain.

This is a rapid and useful method to demonstrate fibrin in tissue.

Krajian stain for fibrin

This is a combination stain to demonstrate elastic tissue, connective tissue, fibrin, and amyloid, employing Congo red. It has been found especially useful to demonstrate fibrin in lungs from unresolved pneumonia. (See elastic tissue stain, p. 1702.)

Mallory iron chloride–hematoxylin for fibrin

1. Prepare frozen or paraffin sections. Stain sections on the slide for 5 min. in 10% aqueous ferric chloride solution.
2. Drain and blot; then pour on a few drops of freshly prepared 1% aqueous solution of hematoxylin. If all the hematoxylin is precipitated by the excess ferric chloride, pour off the solution and add a fresh supply. In 3-5 min. the sections will be colored bluish black.
3. Wash in water.
4. Decolorize and differentiate in 0.25% aqueous ferric chloride. The sections should be kept constantly moving in the solution. Differentiation will be complete within a few seconds to 1 min. or more.
5. Wash in water.
6. Dehydrate in 95% alcohol, followed by absolute alcohol.
7. Clear in oil of origanum for 3-5 min.
8. Clear in 2 changes of xylol, 3 min. each.
9. Mount in gum damar.

Nuclei take a sharp, permanent, dark blue stain; fibrin, grayish to dark blue stain.

AMYLOID STAINS

Amyloid is a firm translucent albuminous substance. In fresh material, Gram's iodine turns amyloid a deep blue. Add a little dilute acetic acid to the preparation before treating with the iodine solution. Treated first with iodine and then with concentrated sulfuric acid, the color of amyloid material will change from brown to blue.

Krajian amyloid stain (modified Bennhold method)

1. Stain unmounted frozen sections in Harris hematoxylin about 15 sec. and wash thoroughly in tap water until they are light blue.
2. Transfer to Krajian Congo red (p. 1662) for 15 min.
3. Wash in tap water.
4. Differentiate in 2% aqueous sodium cyanide solution for a few seconds and wash thoroughly in tap water.
5. Float sections onto slides and allow to drain thoroughly (not dry).
6. Mount in warm glycerin jelly.

Amyloid is stained red and is beautifully differentiated from surrounding tissue. The clearing of the tissue does not in any way interfere with the amyloid stain, and the picture is further enhanced by the hematoxylin nuclear stain. The elastic fibers also are stained red but are of a darker hue and may be easily differentiated from amyloid.

Of the number of staining methods for amyloid, this one is best for brilliant staining and permanent keeping without fading.

Iodine and sulfuric acid reaction

1. Stain sections quickly in dilute Lugol solution.
2. Treat with dilute sulfuric acid (5%) on the slide.

The change of color from red to blue usually occurs within a few minutes but occasionally does not take place at all.

This is not a dependable method for staining amyloid.

Reaction with methyl violet

1. Stain frozen sections in 1% aqueous methyl violet 3-5 min. Rinse in water.
2. Differentiate in 1% aqueous acetic acid.
3. Wash thoroughly in tap water to remove all traces of acid.
4. Mount in glycerin jelly.

The amyloid is stained violet red and the tissue blue. The stain will keep only a few hours and soon fades.

HYALIN STAINS

Hyalin degeneration takes place in the liver, in renal epithelium, in lymph nodes, and in some malignant tumors. Chemically, very little is known about it other than that it is protein in nature.

In fresh specimens it resists dilute acids, alkalies, and alcohol and is not affected by water. It does not give a brown reaction when treated with iodine, nor does it turn blue after treatment with sulfuric acid. In sections it takes an uneven blue color when stained with Krajian elastic and connective tissue stain.

Kuhne modified technic for hyalin

1. Fix tissues in formalin, Zenker fluid, or alcohol.
2. Stain frozen, paraffin, or celloidin sections on the slide for 3 min. in freshly mixed Kuhne crystal violet (p. 1662).
3. Wash in tap water.
4. Treat with Lugol solution for 2 min.
5. Rinse rapidly in tap water.
6. Differentiate in saturated solution of fluorescein in absolute alcohol until the hyaline material stains in various shades of blue and the other constituents decolorize.
7. Rinse in tap water.
8. Counterstain for 2 min. in 1% aqueous neutral red.
9. Rinse in tap water.
10. Dehydrate rapidly in 90% alcohol and then absolute alcohol, controlling the degree of differentiation of the neutral red under a microscope.
11. Clear in xylol and mount in gum damar.

The introduction of neutral red into this method gives an extremely transparent nuclear stain. The hyaline substance stains in shades of blue; the older the hyalin, the darker the blue. This is a rapid and useful stain.

Russell carbolfuchsin method for hyalin

1. Fix tissues by one of the standard fixatives.
2. Cut frozen, paraffin, or celloidin sections and treat them on the slide as follows:
3. Stain in carbolfuchsin for 10-30 min.
4. Rinse in tap water.
5. Pass rapidly through absolute alcohol (½-1 min.).
6. Differentiate and counterstain for a few minutes in the following solution:
   Iodine green . . . . . . 1 gm.
   Phenol, 5% aqueous solution . 100 ml.
7. Rinse rapidly in absolute alcohol.
8. Clear in xylol and mount in gum damar.

Hyaline substance stains bright red; nuclei, light green.

This is a very useful stain.

Weigert fibrin stain for hyalin (Gram-Weigert method)

The Weigert fibrin method stains hyaline material blue black (see Gram-Weigert method, p. 1712). Krajian's connective tissue and hyalin stain, applicable to frozen and paraffin sections of formalin-fixed tissue, stains hyalin bright red (p. 1695).

GLYCOGEN STAINS

Glycogen is a carbohydrate occurring in cells and nuclei, either diffusely or more commonly in large or small granules. It is transparent in appearance and is water-soluble, but it can be precipitated by absolute alcohol.

Tissues to be examined for glycogen should be as fresh as possible since glycogen may be transformed into sugar after death. Tissues removed from the body should first be dried with a towel and then immediately placed in absolute alcohol. After fixation for 1 hr. they should be transferred into fresh absolute alcohol.

There are 2 good methods for staining glycogen: Langhan iodine method and Best carmine method.

Langhan iodine stain for glycogen

1. Fix thin blocks of tissue in absolute alcohol, clear in xylol, and embed in paraffin.
2. Cut paraffin sections; dissolve paraffin in pure xylol and then in absolute alcohol.
3. Stain sections in Lugol solution for 5-10 min.

4. Dehydrate in 1 part tincture of iodine to 4 parts absolute alcohol. Wipe around section rapidly.
5. Clear and differentiate in oil of origanum.
6. Wash in xylol to stop overdifferentiation.
7. Mount in gum damar.

The standard method is to mount the section in oil of origanum or gum damar, but no matter how the sections are mounted the stain tends to fade. Glycogen is stained brownish red; the nuclei, if stained in hematoxylin, are greenish. This is a rapid stain, but it is not permanent.

Best carmine method for glycogen—Krajian modification

1. Fix tissues in absolute alcohol. Embed in either paraffin or celloidin. Celloidin is preferable, since it prevents the glycogen from dissolving in water. If paraffin sections are used they must be dipped twice in a thin celloidin (commonly used for attaching frozen sections) after deparaffinization. The celloidin or modified paraffin sections are then stained in the following manner:
2. Stain sections deeply in Harris or Delafield hematoxylin.
3. Destain if necessary and wash thoroughly in tap water until blue.
4. Mordant in 5% alcoholic benzoic acid for 5 min.
5. Without washing, add an equal volume of Best carmine stain (p. 1659), mix well, and let stand for 5 min.
6. Differentiate in Best differentiator (p. 1659) for 1 min. or more, dipping section in and out until no more pink color is seen.
7. Dehydrate in absolute alcohol or anhydrous isopropanol and then in xylol-acetone solution (1 part acetone to 4 parts xylol).
8. Clear in xylol and mount in gum damar.

Glycogen stains red; nuclei, blue.

This is an excellent and permanent stain for glycogen.

Treatment of pale-staining sections with 1% aqueous basic fuchsin acidified with 0.5% hydrochloric acid will brighten the glycogen stain.

MUCIN STAINS

Mucin is an intercellular secretory product, having some proteid substance. It is soluble in weak alkalies and is precipitated by 1% acetic acid, forming threads or granules.

Hoyer thionine stain for mucin

Tissues may be fixed in one of the standard fixatives, but saturated aqueous solution of mercuric chloride gives the best results.

1. Stain paraffin sections, after freeing them from paraffin, for 10-15 min. in the following solution:

Thionine, saturated aqueous solution	2 drops
Distilled water	5 ml.

2. Dehydrate rapidly in absolute alcohol.
3. Clear in xylol.
4. Mount in gum damar.

Mucin stains purple to red; other constituents, shades of blue. However, mast cell granules, ground substance of cartilage, and jelly of umbilical cord also stain purple by this method. Do not treat the mercury-fixed sections in iodine. This is a useful and permanent method.

Mayer mucicarmine stain—Krajian modification

1. Tissues may be fixed in 10% formalin or Zenker fluid, and frozen or paraffin sections made.
2. Stain sections deeply in Harris or Delafield hematoxylin for about 3 min.
3. Wash in tap water until blue.
4. Stain for 10-15 min. in Krajian modification of Mayer mucicarmine (p. 1660).
5. Rinse and treat with martius yellow for 1 min.
6. Dehydrate in several changes of absolute alcohol or isopropanol and blot in filter paper.
7. Clear in pure xylol and mount in gum damar.

Mucin is stained pink. Cartilage cells also are stained pink, though some claim that this is a specific stain for mucin.

This method is applicable to frozen sections, is easy to perform, and gives brilliant results.

Mucin stain—Krajian carbolfuchsin method

1. Fix tissues in 10% formalin.
2. Prepare frozen or paraffin sections in usual manner.
3. Stain in Krajian carbolfuchsin (p. 1661) for 5 min.
4. Wash in tap water.
5. Differentiate with picric acid-alcohol mixture (equal volumes of saturated aqueous picric acid and 95% alcohol) for 5-10 sec., agitating the slide during this period.
6. Wash thoroughly in tap water.
7. Dehydrate in isopropanol or 95% alcohol for 1 min. and follow with anhydrous isopropanol or absolute alcohol.
8. Blot dry and clear in xylol for 2 min.
9. Mount in gum damar.

Mucin assumes a magenta color and sometimes is pale lilac; nuclei are reddish brown.

The differentiation is very important and should be controlled under a microscope for uniform results.

The staining solution is not permanent. It should be freshly prepared before use.

Pseudomucin

Pseudomucin is dissolved in water, and when treated with 95% alcohol, it is precipitated in threadlike masses, which are again soluble in water.

Pseudomucin is not affected by acetic acid. It is found in certain ovarian tumors.

FAT AND LIPOID STAINS

Fats and lipoids are chemically related substances. They occur in the human body under normal and diseased conditions, generally in mixtures, rarely in pure form. The different fats and lipoids are detected by chemical methods, and large numbers of them may be recognized in color reactions by some staining methods.

Fresh unfixed fat globules are soluble in ether, chloroform, alcohol, xylol, benzene, and other fat solvents. For this reason fixing fluids should be free from these substances. Formaldehyde fixes fat-containing material but does not dissolve its fat globules.

The following important types of fats and lipoids are found in the body: true fats, fatty acids, oils, soaps, cholesterins, cholesterols, waxes, and myelins.

Scharlach R stain for fat—Krajian modification

The most commonly used stain for fat is scharlach R (scarlet red). Sudan III was used originally, but it is less brilliant.

1. Fix tissues in 10% formalin and cut frozen sections in the usual manner.
2. Pass the section quickly through 70% alcohol.
3. Place for 3 min. in Krajian (saturated) scharlach R solution (p. 1662). (Use only the clear portion of the stain to prevent forming a precipitate.)
4. Wash quickly in 70% alcohol.
5. Rinse in tap water.
6. Stain in Harris hematoxylin for 30 sec. and wash in water.
7. Differentiate in 2% aqueous sodium cyanide for 10-15 sec.
8. Wash in water, transfer to a slide, and drain off the water.
9. Mount in warm glycerin jelly.

The addition of benzoic acid to the staining solution materially intensifies the resulting color and prevents deterioration. True fats stain intensely; cholesterins stain less intensely. This is a rapid and permanent stain and is highly recommended.

Osmic acid stain for fat

1. Fix thin slices of tissue, not over 2 mm. thick, in Flemming solution for 1-3 days.

   Flemming solution:

Osmic acid, 2%	4 ml.
Chromic acid, 1%	15 ml.
Glacial acetic acid	1 ml.

2. Wash under running water for 6-12 hr.
3. Dehydrate in 80%, 95%, and then absolute alcohol, about 2 hr. in each.
4. Clear in chloroform. Do not use ether, xylol, or toluol for clearing because they dissolve the fat. Embed in paraffin.
5. Cut sections and dissolve the paraffin with xylol.
6. Mount in chloroform-balsam.

Fat globules stain black to dark gray; lipoidal granules, brownish.

This is a useful and permanent method.

Osmic acid fat stain for frozen sections

1. Fix tissues in 10% formalin for 24 hr. or longer.
2. Cut frozen sections.
3. Place in 1% aqueous osmic acid for 1-2 hr.
4. Wash for 10 min. in several changes of tap water.
5. Place in absolute alcohol or anhydrous isopropanol for 15 min. to obtain the secondary staining of fat not yet colored.
6. Wash in tap water.
7. Mount in glycerin jelly.

Fat globules assume a brown-black color. This is a useful fat stain for frozen sections.

Nile blue sulfate stain for fat—Lorraine Smith

1. Fix tissues in 10% formalin and cut frozen sections.
2. Stain in saturated aqueous Nile blue sulfate for 5-10 min.
3. Wash in water.
4. Differentiate in 1% aqueous acetic acid until the blue color ceases to diffuse from the section.
5. Wash thoroughly in a large basin of water.
6. Mount in glycerin jelly.

This stain has valuable properties. It gives a double stain, coloring neutral fats red; cholesterin ester and cholesterin fatty acids, light red; cerebrosides, light blue; and fatty acids and soaps, deep blue.

Stain for lipoids—Ciaccio

1. Fix thin blocks of tissue for 2 days in the following solution:

Potassium dichromate, 5%	80 ml.
Formaldehyde (commercial, 40%)	20 ml.
Glacial acetic acid	5 ml.
or formic acid	4 to 5 drops

2. Treat in 3% aqueous potassium dichromate for 2 days.
3. Wash under running water overnight.
4. Dehydrate in graded alcohols, beginning with

70%, and reduce the time in 95% and absolute alcohol as much as possible.
5. Clear in xylol for 1 hr.
6. Place in xylol and paraffin mixture for 1½ hr. in a paraffin oven at 55° C.
7. Transfer to pure paraffin for 1½ hr. in a paraffin oven; then embed.
8. Cut sections and affix to slides.
9. Dissolve the paraffin with 2 changes of xylol.
10. Wash in 2 changes of absolute alcohol.
11. Stain in saturated solution of Sudan III (95 ml. 85% alcohol plus 5 ml. acetone). Stain in a stoppered slide jar for 30-60 min. at 30° C.
12. Differentiate very rapidly in 50% alcohol.
13. Wash in water.
14. Counterstain in Harris hematoxylin for 5 min.
15. Wash in water until section assumes a blue color.
16. Mount in warm glycerin jelly.

The lipoid substances appear as fine orange granules; the nuclei, blue black. This method gives a positive stain for lipoid granules.

MYELIN OR MEDULLARY SHEATH STAINS

It has been stated that cerebrosins and lecithins constitute the normal myelin sheath.

Weigert-Pal method for myelin

1. Fix tissues in 2.5% potassium dichromate for several weeks in an incubator; or fix in 10% formalin first and then treat with potassium dichromate solution.
2. Wash in running water overnight.
3. Dehydrate in graded alcohols, clear, and embed in either paraffin or celloidin.
4. Cut sections 10-20μ thick.
5. Stain in Weigert-Pal lithium-carbonate-hematoxylin mixture (p. 1659) for 24-48 hr.
6. Wash sections in water and treat with 0.25% aqueous potassium permanganate for ½-3 min.
7. Wash in water.
8. Treat sections in the following differentiating fluid for ½-3 min.:
   Oxalic acid, 1% aqueous . . . 1 part
   Potassium sulfite, 1% aqueous . 1 part
   Differentiation is complete when the white matter of the brain tissue is blue black and the gray matter is practically colorless.
9. Wash sections in water and counterstain in lithium carmine or safranin if desired.
10. Dehydrate in absolute alcohol, clear in carbol-xylol and then xylol, and mount in gum damar.

By this method myelin sheaths appear black; recent areas of degeneration, pale, gray; old ones, colorless.

We have omitted the original Weigert method and have replaced it with Pal's modification, which is more rapid and gives sharper myelin sheaths. This is a slow but very useful method.

Spielmeyer method for myelin sheath

1. Fix tissues in 10% formalin 24 hr. or longer.
2. Wash in running water.
3. Cut frozen sections 20-30μ thick.
4. Place in 2.5% aqueous iron ammonium sulfate for 6 hr.
5. Rinse in water; then place in 70% alcohol for 10 min.

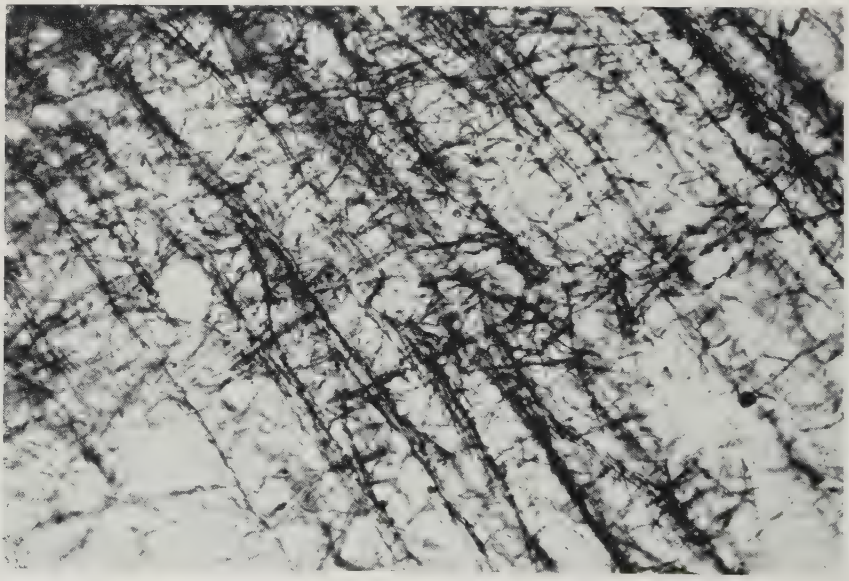


Fig. 558. High-power magnification of myelin sheath. Spielmeyer method. Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

6. Stain sections for 10-24 hr. in Spielmeyer hematoxylin (p. 1659).
7. Rinse sections in water.
8. Differentiate in a weaker solution of iron ammonium sulfate.
9. Wash thoroughly in tap water.
10. Dehydrate in absolute alcohol.
11. Clear in xylol and mount in gum damar.

The hematoxylin solution must be old and should be used over and over, that is, the used portion should be filtered back into the original bottle. The staining power improves with age.

The washing in 70% alcohol removes fatty substances which might interfere with even staining. Occasional irregular staining indicates that the fat has not been entirely removed. Such sections may be carried back through water to 70% alcohol and again stained and differentiated.

This is a good stain to show the fine detail of myelinated fibers.

Krajian myelin sheath stain

1. Fix tissues in 10% formalin. Cut frozen sections 10-15μ thick.
2. Place sections in **4% Congo red in glycerin** for 10 min. (Congo red 4%, 8 ml.; glycerin, 2 ml.).
3. Wash thoroughly in tap water.
4. Place in 2% aqueous potassium iodide for 2 min.
5. Wash and place in 3% aqueous sodium cyanide for 2 min.
6. Wash thoroughly in tap water.
7. Place in 10% aqueous ferric ammonium sulfate for 10 min. at 55° C.
8. Wash in tap water.
9. Place in 2% ripe hematoxylin solution for 5 min. (2 ml. ripe hematoxylin, p. 1659; 8 ml. distilled water).
10. Wash in tap water.
11. Destain in 2% aqueous ferric ammonium sulfate until the section appears light blue, about 30-45 sec.
12. Wash thoroughly in tap water.
13. Differentiate in 0.15% aqueous potassium permanganate for 10-20 sec.
14. Wash in tap water.
15. Place in 3% aqueous sodium cyanide until sections flatten and assume a light blue appearance.
16. Wash in tap water and attach to a glass slide.
17. Dehydrate in 2 changes of absolute alcohol.
18. Clear in oil of origanum for 2 min.
19. Clear in pure xylol for 2-5 min.
20. Mount in gum damar.

This method has all the advantages of the longer methods and shows very fine details of the fibrils, yet it can be completed in 1 hr. It is highly recommended.

Pal-Kulschitzky method for myelin sheath

1. Fix tissues in 10% formalin for a few days or longer and place them in the following solution for 4-5 days or longer at room temperature:

Potassium dichromate . . .	5 gm.
Fluorchrome	2 gm.
Distilled water	100 ml.

2. Pass the blocks through graded alcohols for dehydration and then in ether-alcohol mixture, thin celloidin, thick celloidin, and embed.
3. Cut sections 35μ thick or thicker and place in 80% alcohol.

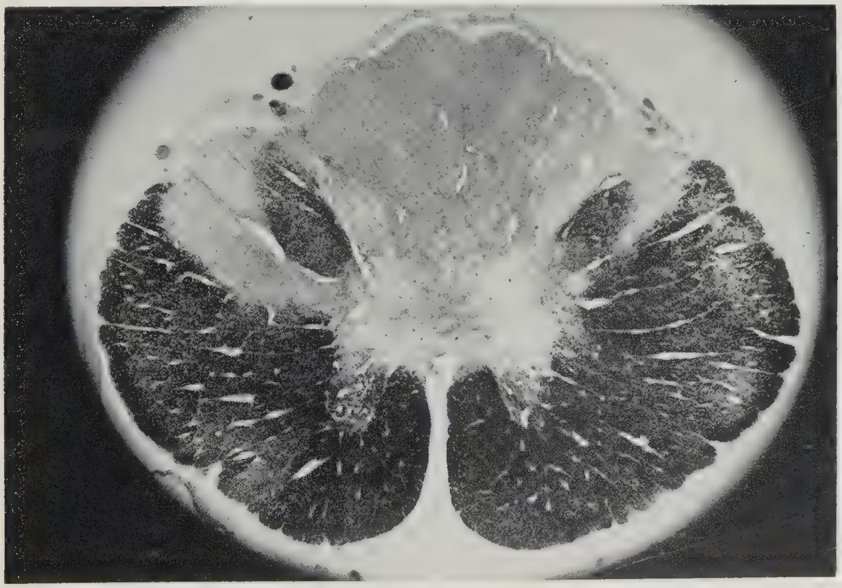


Fig. 559. Tabes dorsalis. Posterior column degeneration of myelin sheath in spinal cord. Krajian myelin sheath method. Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

4. Stain sections at 37° C. for 24 hr. in Pal-Kulschitzky hematoxylin (p. 1659).
5. Wash sections individually in tap water.
6. Place in 0.3% aqueous potassium permanganate about 1 min. or longer.
7. Wash in distilled water.
8. Differentiate in equal parts of 1% aqueous oxalic acid and 1% aqueous potassium sulfite.
9. Wash in tap water, in which the sections can be kept for any length of time. If differentiation is unsuccessful, the sections may be carried back from water into potassium permanganate solution and from there again to water and oxalic-potassium sulfite solution. This procedure can be carried out several times until differentiation is completed.
10. Dehydrate in graded alcohols, clear in pure xylol, and mount in gum damar.

Wolter modification of Pal-Kulschitzky myelin sheath stain

Wolter has modified this method so that it may also be used for frozen sections.

1. Cut frozen sections about 40μ thick and place them in 70% alcohol; then place in dichromate and fluorchrome solution for 24 hr. at 37° C. (p. 1709).
2. From the latter (the mordanting fluid) pass the sections through 80% alcohol to the hematoxylin solution and keep in hematoxylin 24 hr. at 37° C.
3. Staining is completed as above, commencing with the washing after hematoxylin.

This method usually yields satisfactory results and is valuable for rapid staining of myelin sheaths.

Morgan myelin stain for paraffin sections

Morgan's method of staining myelin is useful in fresh as well as in old formol-fixed material.

1. Fix brain tissue in 10% formalin, embed in paraffin, and section.
2. Mordant sections for 2 hr. in 4% aqueous iron alum (ammonium ferric sulfate).
3. Stain 8-16 hr. in well-ripened Heidenhain hematoxylin (p. 1658).
4. Destain in 2% iron alum for a few minutes until the larger fiber tracts become well outlined.
5. Rinse in tap water.
6. Complete the differentiation in 0.5% hydrochloric acid. In a few seconds the background becomes colorless and the fibers well differentiated.
7. Wash sections in running water for at least 1 hr. to remove acid and alum.
8. If desired, counterstain with well-ripened neutral red.
9. Dehydrate, clear in xylol, and mount in gum damar or balsam.

In successful preparations the myelinated fibers stain dark blue, the background clear and white. For destaining with 2% alum the process should be stopped by placing the sections in tap water before all the blue of the hematoxylin has been extracted from the background, and the treatment with the hydrochloric acid should be very brief and carefully watched. When the proper stage has been reached, stop the decolorizing by transferring the sections to tap water.

Scharlach R stain for demonstration of myelin sheath

The improved scharlach R solution, ordinarily used to demonstrate fat, may be successfully employed to bring out myelin sheath in the tissues of the central nervous system, especially in spinal cords (see scharlach R stain for fat, p. 1662).

XANTHYDROL METHOD FOR UREA CRYSTALS[31]

The following technic, originated by Krajian,[32] is useful in differentiating at autopsy actual uremic from nonuremic cases. In other words, it has been found that patients dying with uremia have urea crystals in the brain tissue, whereas patients having a disease simulating uremia do not have urea crystals in the brain tissue.

Regular method

1. Cut thin blocks of tissue 2-3 mm. thick. Immerse 5-6 hr. in a freshly prepared solution of xanthydrol (5 gm. xanthydrol in 100 ml. glacial acetic acid).
2. Wash under running water for 1 hr.
3. Fix in dilute solution of formalin (1:10) 6 hr. or longer. In urgent cases rapid fixation with heat may be used.
4. Wash in tap water.
5. Cut frozen sections 10μ thick.
6. Transfer to a glass slide.
7. Pour several drops of dehydrated alcohol over the section from a drop bottle and blot. Repeat this process once more.
8. Dip in thin celloidin (p. 1666) in the usual manner.
9. Blow the breath over the section.
10. Stain in 1% aqueous eosin for several minutes.
11. Wash in tap water.
12. Dehydrate in 3 changes of dehydrated alcohol from a drop bottle.
13. Place in carbolxylol for 2 min.
14. Clear in 2 changes of pure xylol, 1 min. in each.
15. Mount in gum damar.

Because of the high diffusibility of urea, the tissue must be kept from contact with any fluid prior to its immersion in xanthydrol. One should especially avoid contamination of the

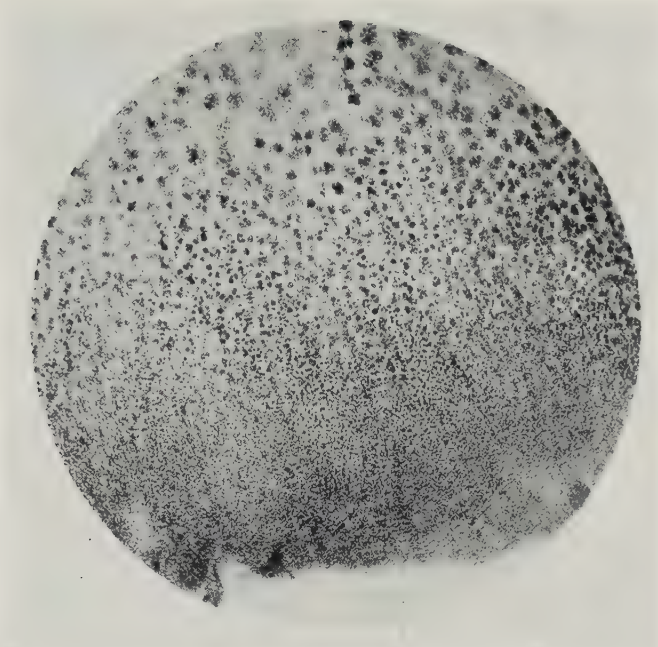


Fig. 560. Xanthydrol method for urea by regular method (6 hr. fixation). Low-power magnification. Krajian method. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

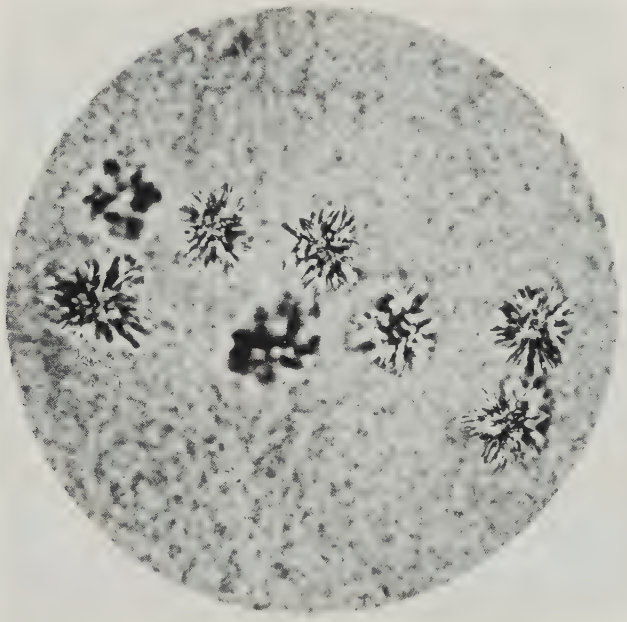


Fig. 561. Xanthydrol method for urea. Rapid method 1½ hr. fixation in an oven at 70° C. High-power magnification. Krajian method. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

tissue with urine during the autopsy. The blocks must be very thin since the rate of penetration by the xanthydrol-acetic acid solution is slow. The use of hematoxylin to stain the nuclei tends to disturb the crystals; accordingly, it is preferable merely to stain the background with eosin.

Rapid xanthydrol method

1. Fix thin blocks in hot 5% xanthydrol solution 1½ hr. in an oven at 70° C.
2. Wash in tap water for 1-2 min.
3. Fix in hot 10% formalin for 15 min. at 70° C.
4. Rinse in tap water.
5. Cut frozen sections as superficially in the block as possible since penetration by the xanthydrol is very shallow.
6. Stain and mount as in the longer method.

Smear method for xanthydrol test

For very rapid, approximate, or preliminary diagnosis, use the following method:

1. Boil very small bits of brain tissue in a test tube for 2 min. in 5% xanthydrol solution.
2. Smear tissue on a glass slide.
3. Flood with absolute alcohol 2 or 3 times.
4. Stain as in the other methods.

STAINING METHODS FOR MICROORGANISMS IN TISSUE SECTIONS

Several methods have been devised or modified for demonstrating gram-positive and gram-negative organisms, but the Gram-Weigert stain remains the method of choice.

Gram-Weigert method of staining microorganisms in tissue

1. Fix tissues in 10% formalin and cut very thin paraffin, celloidin, or frozen sections and mount each in the usual manner.
2. If paraffin sections are made, remove paraffin with 2 changes of xylol and then 2 changes of absolute alcohol.
3. Stain in 1% aqueous eosin for 5 min.
4. Wash in water.
5. Stain in Sterling gentian violet for 3 min. for frozen sections or for 10 min. for paraffin sections.
6. Wash off with Gram's iodine solution and flood with the same solution for 3 min.
7. Blot in filter paper.
8. Decolorize with several changes of equal parts of aniline oil and xylol until color ceases to wash out.
9. Clear in 2 changes of xylol, blotting after each change.
10. Mount in gum damar.

As a counter stain, a weak carbolfuchsin or 1% aqueous neutral red may be employed instead of 1% eosin.

Gram-positive organisms and fungi stain a beautiful violet color, but gram-negative organisms are not ordinarily seen.

MacCallum modification of Goodpasture method for demonstrating gram-negative organisms in tissue

1. Fix tissues in Zenker-formalin solution (Helly fluid), embed in paraffin, and cut very thin sections, mount, and carry through the usual process to water.
2. Stain 10-30 min. or longer in Goodpasture fuchsin solution, formula 1 (p. 1661).
3. Wash in water.
4. Differentiate in 40% formaldehyde. Only a few seconds are required. The bright red color changes to rose.
5. Wash in water.
6. Counterstain in saturated aqueous picric acid for 3-5 min. until the section becomes purplish yellow.
7. Wash in water.
8. Differentiate in 95% alcohol. The red reappears and some of it is washed out, as is also some of the yellow of the picric acid.
9. Wash in water.
10. Stain in Sterling gentian violet (p. 1661) for 5 min. or longer.
11. Wash in water.
12. Mordant in Gram's iodine for 1 min.
13. Blot dry without washing.
14. Clear in a mixture of equal parts of aniline and xylol until color ceases to wash out.
15. Clear in 2 changes of xylol.
16. Mount in gum damar.

Gram-negative bacteria stain red; gram-positive bacteria, blue; tissue, red and blue; fibrin, deep blue. This is a useful stain to demonstrate gram-negative organisms.

Application of Gram stain to paraffin sections—Glynn[33]

1. Bring paraffin sections through xylol and alcohol to water in the usual manner.
2. Stain with carbol gentian violet for 2 min.
3. Drain but do not wash.
4. Apply Gram's iodine for 1 min.
5. Apply acetone until no more color is removed, about 10-15 sec.
6. Wash in water. Do not allow the section to dry.
7. Apply 0.05% basic fuchsin in 0.05N hydrochloric acid for 3 min.
8. Drain but do not wash.
9. Apply saturated aqueous picric acid for 30 sec.-1 min.
10. Wash in water.
11. Differentiate and dehydrate in acetone for 10-15 sec.
12. Clear in xylol.
13. Mount in balsam.

A staining rack and dropping bottles rather than staining jars are recommended since these permit use of uncontaminated reagents.

Preliminary fixation of tissue can be accomplished by the common fixatives; formaldehyde solution (1:10), Zenker fluid, Zenker fluid plus dilute solution of formaldehyde, or Bouin fixative can be used with success. Better histologic detail seems to be obtained by the use of Zenker fixative without acetic acid.

When viewed under the low power of the microscope, the tissue is lightly stained. Gram-positive bacteria are deep violet and easily identified under low power. When cellular exudation is not too abundant, the deep red gram-negative bacteria can also be seen under low power in contrast to the lighter red nuclei. Under oil immersion both types are sharply differentiated.

Nuclear chromatin stains red; cytoplasm, faint yellow. The nuclei of nerve cells show differentiation of nucleoli and Nissl bodies. Axons are unstained. Myelin sheaths are violet. Fibrin and the fibers of connective tissue are pale pink. Erythrocytes are yellow.

Gram-positive bacteria are deep violet. Staphylococci, streptococci, mycelia of actinomycetes, and vegetative forms of spore-bearing species are solidly stained. Diphtheria bacilli show barred and granular forms. Human tubercle bacilli are less deeply stained than other gram-positive organisms and show distinct beading.

Gram-negative bacteria are uniformly red. The clubs of actinomycetes are also red and contrast sharply with the gram-positive mycelium.

The chief advantage of this stain is that it not only provides simultaneously good histologic and bacterial differentiation but it also stains all bacteria that may be present. This is particularly useful in the study of tuberculous tissue where very young tubercle bacilli may not be acid-fast and secondary invaders are easily identified.

Krajian method of demonstrating gram-negative organisms in tissue

1. Prepare thin frozen or paraffin sections (5-7μ) in the usual manner and bring them down to water.
2. Mordant in 5% aqueous thorium nitrate for 5 min.
3. Rinse in tap water.
4. Stain 3 minutes in Krajian modification of MacCallum hematoxylin (p. 1658).
5. Wash in tap water.
6. Decolorize in 1% acid alcohol until the excess color ceases to come off.
7. Wash in tap water.
8. Treat with a mixture consisting of equal volumes of 10% potassium iodide and 10% zinc sulfate for 5 min.
9. Rinse in tap water.
10. Counterstain with carbolfuchsin (Ziehl-Neelsen) for 7-10 min.
11. Wash in tap water.
12. Treat with 5% sodium sulfite for 2 min.
13. Rinse in water.
14. Treat with 3% acetic acid for 3 min.
15. Blot and apply aniline-xylol (equal volumes of aniline oil and xylol) for 30 sec.
16. Treat with pure creosote for 2-5 min., agitating the section for even destaining and clearing. Stop when no more pink color is seen.
17. Clear for 2 min. in pure xylol and mount in gum damar.

Gram-positive organisms assume hematoxylin color; gram-negative organisms, bright red.

The thorium nitrate solution should be prepared fresh.

Rapid staining method for gram-positive and gram-negative organisms in frozen and paraffin sections[34]

Christian Gram[35] in 1884 devised his method for demonstrating bacteria in tissue sections. Weigert's[36] modification of Gram's method has been useful for demonstrating fibrin and bacteria and is still widely used, but both methods demonstrate only gram-positive organisms. Numerous attempts have been made to demonstrate both gram-positive and gram-negative organisms in paraffin sections of tissues that had been fixed in Zenker solution or in formalin, and methods accomplishing this have been reported by MacCallum,[37] Lyon,[38] Lillie,[39] Brown and Brenn,[40] Rudnikoff and Stawsky,[41] Glynn,[33] and others.

In these modifications the staining solutions as devised by Gram remained practically unchanged. No attempt had been made to devise a new method using some other dye or chemical, and no recommendations had been made as to the applicability to frozen sections.

The solutions used by Gram and those used in the modifications of his method are gentian violet, methyl violet, and crystal violet as gram-positive stains, Gram solution and compound solution of iodine (Lugol solution) as differentiators; rosaniline hydrochloride, basic fuchsin, dilute carbolfuchsin, and safranin as gram-negative stains, and acetone, alcohol, ether, and aniline oil-xylol as decolorizers.

The need for rapid diagnosis led to investigation of the usefulness of a different dye

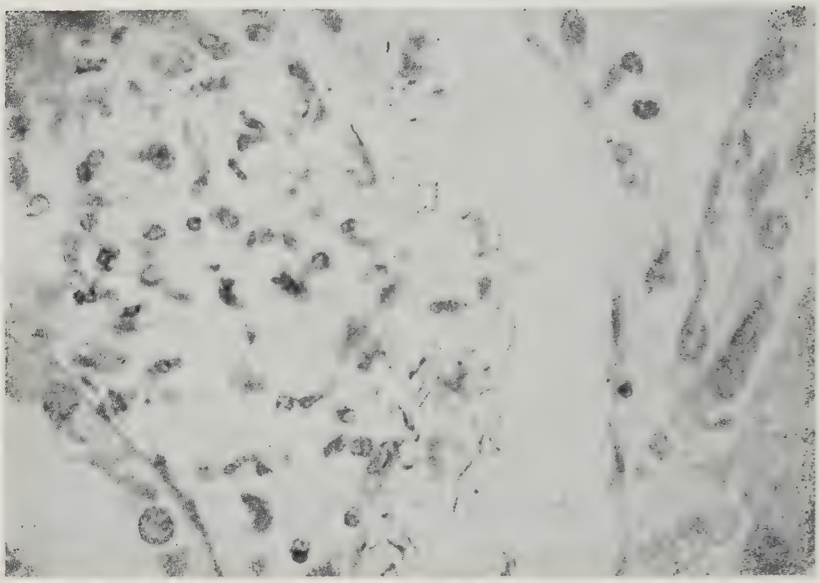


Fig. 562. Streptococci in kidney, stained blue. (Krajian stain; ×1620.) (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

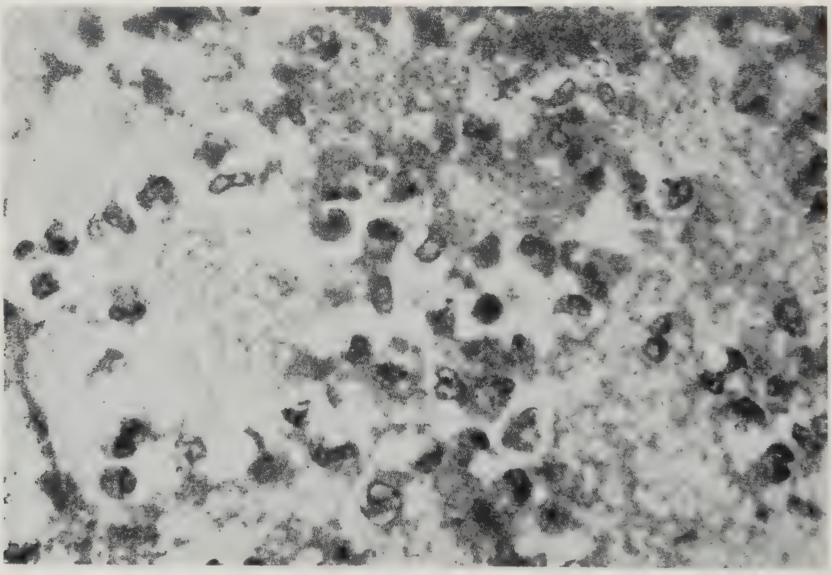


Fig. 563. *Klebsiella* in pneumonia, stained red. (Krajian stain; ×1620.) (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

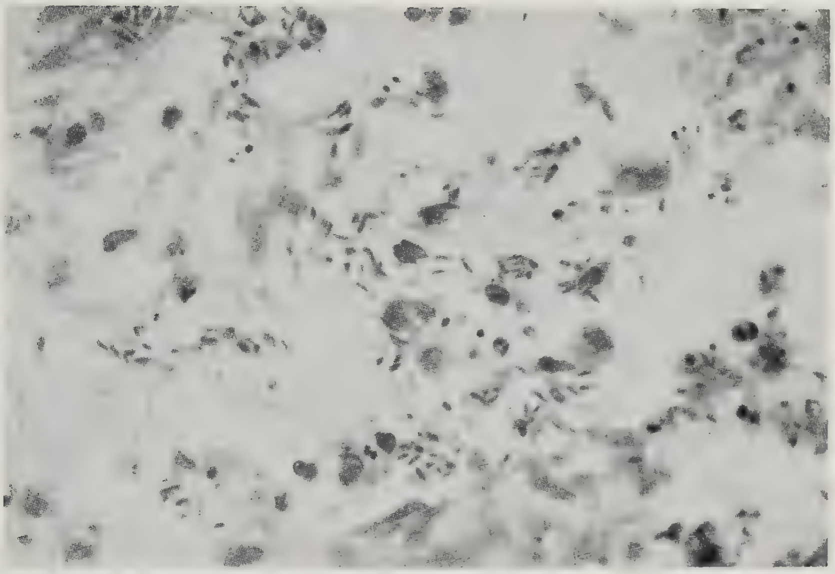


Fig. 564. *Sporotrichum* in testicle, stained blue. (Krajian stain; ×1620.) (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

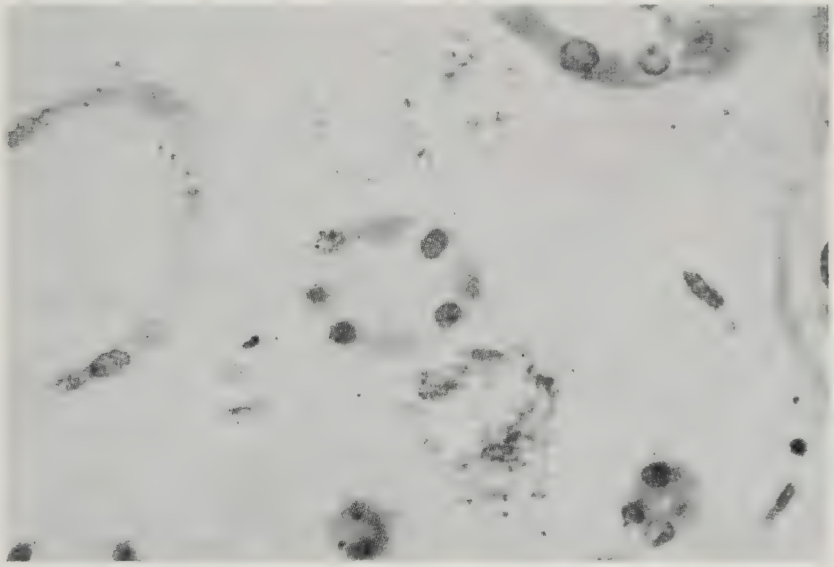


Fig. 565. *Escherichia coli* in kidney tubules, stained red. (Krajian stain; ×1620.) (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

and different solutions. A simple method was devised which is easily applied to frozen and paraffin sections of formalin-fixed tissue and which gives satisfactory simultaneous differential staining of gram-positive and gram-negative organisms. For example, 4 different sections (frozen or paraffin) mounted on the same slide, one each of a kidney containing *Candida,* a lung with *Klebsiella,* a testicle containing *Sporotrichum,* and a kidney with *Escherichia coli,* were stained, with successful differentiation of the organisms by the method described. The technic has also been used on tissues containing streptococci, *Clostridium perfringens, Haemophilus influenzae, Actinomyces,* meningococci, gonococci, *Corynebacterium diphtheriae,* and Negri bodies.

The solutions used are methylene blue as a gram-positive stain, fuchsin-creosote as a gram-negative stain, and creosote-xylene as a differentiator.

Frozen section method for staining gram-positive and gram-negative organisms in tissue

1. Prepare frozen sections 7-10μ thick and mount in the usual manner under thin celloidin (p. 1666).
2. Stain for 3 min. with Loeffler methylene blue.
3. Wash in tap water and dehydrate rapidly with 3 applications of anhydrous isopropanol or absolute ethyl alcohol.
4. Differentiate rapidly with **creosote-xylol** (1 part creosote plus 2 parts xylol), agitating the slide constantly for 5-10 sec.
5. Pour off and apply **creosote-fuchsin** (2.5 ml. 6% alcoholic basic fuchsin added to 50 ml. creosote-xylol), agitating the slide about 15-20 times, changing the solution once.
6. Blot and apply creosote-xylol 2 or 3 times, agitating the slide constantly for even decolorization or until most of the excess red color leaves the section.
7. Blot, clear for 2 min. in xylol, and mount in gum damar.

Paraffin section method for staining gram-positive and gram-negative organisms in tissue

1. Deparaffinize sections with 2 applications of xylol and 2 applications of absolute alcohol or isopropanol.
2. Hydrate to tap water and then proceed with the method described above.

Nuclei are red; gram-positive organisms, blue; gram-negative organisms, red; devitalized gram-positive organisms, red; *Candida* and *Actinomyces,* blue; Negri bodies, bright red with bluish chromatin bodies; and fibrin, sometimes blue and sometimes red.

All the staining solutions are stable.

ACID-FAST STAINS

Ziehl-Neelsen method of staining acid-fast organisms in tissue

The Ziehl-Neelsen method is the standard method of staining acid-fast bacilli in sections. It is used to stain both tubercle and Hansen bacilli.

1. Fix tissues in Zenker fluid or formalin and make paraffin or frozen sections. Mount frozen sections as usual.
2. Remove paraffin with 2 changes of xylol and absolute alcohol.
3. Stain lightly in Harris hematoxylin.
4. Wash in tap water until blue.
5. Stain in carbolfuchsin over a Bunsen burner for 10 min., gently steaming. This method is used for rapid diagnosis; better results are obtained if sections are left in carbolfuchsin solution 16-24 hr. at room temperature.
6. Wash in water.
7. Decolorize a few seconds in 1% hydrochloric acid in alcohol.
8. Continue the decolorization in 70% alcohol until no more pink color is seen.
9. Dehydrate in absolute alcohol; then blot.
10. Clear in 2 changes of xylol.
11. Mount in gum damar.

Do not use carbolxylol for clearing since it causes the red color of the bacilli to fade.

Stain Hansen bacilli only 5 min. by the steaming method and decolorize 15 sec. in acid alcohol.

Hansen and tubercle bacilli appear bright red; nuclei of cells take the hematoxylin stain.

Krajian method of demonstrating acid-fast organisms in tissue sections[42]

The importance of a consistently satisfactory and dependable method for identification of tubercle and Hansen bacilli and of allied acid-fast organisms in the granulomatous lesions of biopsy and necropsy material cannot be overemphasized. The methods described in microtechnics call for application of a nuclear stain first, then carbolfuchsin (Ziehl-Neelsen) for the staining of organisms, then a decolorizer, and finally dehydrating and clearing.

The unsatisfactory feature in these accepted standard methods has always been in the use of the decolorizer. Carbolfuchsin not only stains the organisms but it also is absorbed by the connective tissue system. If the decolorizer (acid alcohol) is overapplied to remove the color from the connective tissue fibers, it will also affect the color of the organism and fade the nuclear stain. To overcome this obstacle there has been developed a satisfactory and dependable method, making changes in the type of nuclear stain and decolorizer and introducing a differentiating step. The method is easily applied to frozen and paraffin sections fixed in ordinary fixatives.

1. Cut frozen sections 7-10μ thick.
2. Transfer to a glass slide, dehydrate, blot in filter paper, and dip once in thin celloidin (p. 1666); blow over the surface until dry.
3. Wash in tap water until the surface is wet.
4. Place on a tripod or on a warming table, apply carbolfuchsin, and steam gently with a flame for 3 min.
5. Discard the staining solution, wash with tap water, and apply **arsenic acid alcohol** (1 gm. arsenic acid in 100 ml. 60% ethyl alcohol) with a medicine dropper 2-4 times, agitating the slide constantly for even destaining or until most of the red color is removed.
6. Wash in tap water and apply Loeffler methylene blue solution for 2 min.
7. Wash in tap water and dehydrate with 3 applications of anhydrous isopropanol or absolute ethyl alcohol.
8. Immediately apply iso-creosote (equal parts of anhydrous isopropanol or absolute ethyl alcohol and beechwood creosote) 2 or 3 applications, agitating the slide constantly for even differentiation. This removes the excess blue stain from tissue as well as from organisms. This is the crucial step; if overdifferentiated, the background will be very light blue without affecting the organisms; light blue is the correct color.
9. Blot immediately in filter paper.
10. Clear in 3 changes of xylol, 1 min. each.
11. Mount in gum damar.

For **paraffin sections,** remove paraffin with 2 applications of xylol, 2 min. each, remove xylol with 2 applications of isopropanol or absolute alcohol, wash in tap water, and proceed with the method described.

Figs. 566 and 567 demonstrate the results obtained with this method.

Acid-fast stain of paraffin sections—Fuller

This method is excellent for photomicrographic purposes—tubercle bacilli are stained brilliantly red, whereas nuclei take various

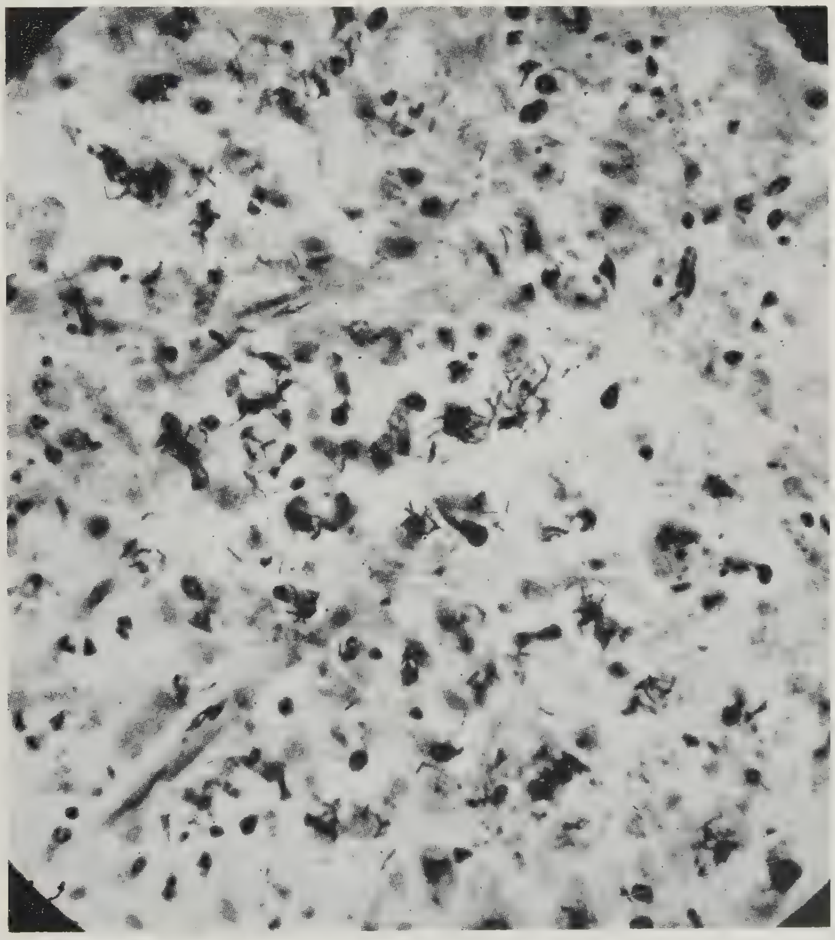


Fig. 566. Tubercle bacilli in lung tissue from a case of miliary tuberculosis. (Krajian acid-fast stain; ×1620.) (From Krajian, A. A.: Am. J. Clin. Path. [Tech. Supp.] 7:45, 1943.)

Fig. 567. Hansen bacilli in fibrous tissue from a case of Hansen's disease of the arm. (Krajian acid-fast stain; ×1620.) (From Krajian, A. A.: Am. J. Clin. Path. [Tech. Supp.] **7**:45, 1943.)

shades of green, so that good contrast is obtainable.

1. Deparaffinize sections in the usual manner and hydrate to water.
2. Mordant in 5% iron alum in a paraffin oven for 5 min. at 45°-50° C.
3. Rinse in water.
4. Stain in Fuller hematoxylin (p. 1659) in a paraffin oven for 5 min.
5. Place directly in the picric acid solution (2 parts saturated alcoholic picric acid and 1 part 95% alcohol) for 5 min. or longer if necessary, until the stain remains only in the nuclei.
6. Wash thoroughly under running water, 15 min. or longer, or until sections are free of picric acid.
7. Place section on heating bar or warming table and pour Fuller aniline fuchsin (p. 1661) over them. Heat until steam comes off, but do not boil.
8. Decolorize in acid alcohol for a few seconds, until the fuchsin begins to come off in clouds; rinse in tap water and continue decolorization until the sections are barely pink. (Acid alcohol is prepared by mixing 3 ml. concentrated hydrochloric acid with 100 ml. 95% alcohol.)
9. Rinse in 1% ammoniated water for a few seconds.
10. Wash thoroughly under running water for 10 min.
11. Stain in 1% light green for 5 min.
12. Wash in water, and dehydrate rapidly in 95% alcohol, followed by absolute alcohol.
13. Clear in xylol for 5 minutes and mount in gum damar.

Krajian modification of Ziehl-Neelsen stain

1. Stain thin frozen, paraffin, or celloidin sections lightly in Harris hematoxylin.
2. Wash in tap water until blue.
3. Stain for 5 min. with carbolfuchsin, gently steaming. Keep the slide fully covered with the staining solution.
4. Wash in tap water.
5. Destain in picric acid alcohol (1 part saturated aqueous picric acid and 1 part 95% alcohol) for 15-30 sec., changing the solution

2 or 3 times and agitating the section for even destaining.

6. Wash thoroughly in a large basin of tap water.
7. Treat with 2% sodium cyanide for 10-15 sec. and wash thoroughly in tap water.
8. Dehydrate completely with several changes of absolute alcohol or anhydrous isopropanol (this also removes the excess fuchsin stain).
9. Blot dry and clear in 2 changes of pure xylol, 2 min. each.
10. Mount in gum damar.

Do not use carbolxylol for clearing because it fades the red color of the bacilli.

Tubercle and Hansen bacilli appear bright red; the nuclei, blue.

Flexner method of staining Hansen bacilli in tissue

1. Stain sections in Harris hematoxylin to obtain a sharp nuclear stain.
2. Wash in water.
3. Stain in Ziehl-Neelsen carbolfuchsin for 2-5 min., steaming, or for 30-60 min. at room temperature.
4. Wash in water.
5. Treat with Gram's iodine ½-1 min.
6. Wash in water.
7. Blot in filter paper, clear, and differentiate in aniline oil.
8. Clear in pure xylol. Mount in gum damar.

Hansen bacilli stain red; nuclei, light blue.

Kinyoun carbolfuchsin method for demonstrating tubercle bacilli in tissue

Solution 1:

Basic fuchsin	2 gm.
Phenol crystals	8 gm.
Alcohol, 95%	20 ml.
Distilled water	100 ml.

Solution 2:

1. Prepare a 0.5% solution of acetic acid by adding 0.5 ml. glacial acetic acid to distilled water and diluting to 100 ml. with distilled water.
2. Dissolve 0.5 gm. methylene blue in this 0.5% acetic acid.

Solution 3:

1. Add 15 ml. hydrochloric acid to 95% alcohol.
2. Dilute to 100 ml. with 95% alcohol.

Procedure:

1. Remove the paraffin from the sections by passing them through 3 changes of xylol in the usual manner.
2. Place slides for 1 min. each in the following solutions: absolute alcohol, 95% alcohol, 70% alcohol, distilled water.
3. Stain with Kinyoun carbolfuchsin for 1 hr. at room temperature.
4. Rinse with distilled water.
5. Decolorize in the hydrochloric acid solution until pink.
6. Counterstain in the methylene blue solution for 1 min.
7. Wash in distilled water.
8. Dehydrate in the usual manner.
9. Mount in balsam, gum damar, or Clarite.

Fluorescence method of demonstrating Mycobacterium tuberculosis in tissues—Tanner

The use of fluorescence in microscopy for demonstrating *Mycobacterium tuberculosis* in smears has been considered on p. 597. The use of fluorescence microscopy for demonstrating this organism in tissues has been reported in European literature.[43-45] Tanner[46] gives an excellent method, using this technic for the demonstration of tubercle bacilli in tissue.

Reagents:

1. Auramine stain.
    (a) Solution A.

Distilled water	87 ml.
Phenol	3 ml.

    (b) Solution B.

Ethyl alcohol, 95%	10 ml.
Auramine-O	0.1 gm.

Add solution B to solution A.

2. Decolorizing solution.

Alcohol, 70%	100 ml.
Hydrochloric acid, c.p., concentrated	1 ml.
Sodium chloride, c.p.	0.5 gm.

3. Methylene blue, 1% solution.
    (a) Dissolve 100 mg. methylene blue in 100 ml. distilled water, grinding in a mortar, and adding small portions of the solvent until all the dye is in solution.
    (b) Filter after 24 hr.

Procedure:

1. Cut sections of tissue blocks, fixed in 10% formalin and embedded in paraffin, at 5μ. Mount on clean glass slides with a minimal amount of egg albumin. When the sections are dry, remove the paraffin in the usual manner and cover the deparaffinized sections with the auramine staining solution for 5 min.
2. Wash the sections and cover with the decolorizing solution for 20 min.
3. Wash with tap water and cover with fresh decolorizer for another 20 min.
4. Wash with tap water and counterstain with 0.1% aqueous methylene blue for 1 min.
5. Wash. Dry. Examine with a fluorescence microscope without using a cover glass.

The methylene blue gives a black or dark blue background but does not interfere with the fluorescence of the organisms.

Some sections, according to Tanner, gave satisfactory results without the methylene blue, but the use of that counterstain gives better contrast and is recommended as a routine procedure in tissue sectioning. The results with both methods are satisfactory, the bacilli standing out as bright yellow rods on a nearly black background. The organism can be identified readily by using an 8 mm. objective and a ×25 ocular.

GIEMSA STAINING OF TISSUE

Giemsa stain for bone marrow study

1. Fix very thin pieces of bone marrow in Zenker fluid for 12-16 hr. Wash under running water for 16-24 hr. Embed in paraffin in the usual manner and cut sections 5μ or thinner.
2. Deparaffinize with 2 applications of xylol, 2 min. each.
3. Remove xylol by 2 applications of absolute alcohol.
4. Treat with tincture of iodine for 5 min. to remove the bichloride of mercury precipitate.
5. Treat with 1% aqueous sodium thiosulfate for 5-10 min.
6. Wash thoroughly in tap water followed by neutral distilled water.
7. Prepare a **dilute Giemsa stain** as follows:

Giemsa stock solution . . .	3 ml.
Methyl alcohol (Merck) . .	3 ml.
Neutral distilled water (add 2 drops 1% sodium bicarbonate to 100 ml. distilled water)	100 ml.

8. Place sections in dilute Giemsa solution for 1 hr. Change the stain twice and leave in the third solution overnight.
9. Place sections in distilled water.
10. Differentiate each slide separately in 95% alcohol to which a few drops of colophonium in absolute alcohol are added.
11. Dehydrate in 2 changes of absolute alcohol.
12. Clear in neutral xylol and mount in cedarwood oil or gum damar.

Nuclei of cells and neutrophil granules stain red violet; basophil granules, blue violet; cytoplasm of lymphocytes, blue; eosinophilic granules and red blood cells, pale red.

Refer to hematology section for other procedures for Giemsa stain of bone marrow.

Giemsa stain for protozoologic and hematologic work

For the demonstration of protozoa and bacteria fix thin pieces of tissue in mercuric alcohol (4 gm. mercuric chloride in 95% alcohol) for 2 days, changing the fixing solution after 24 hr.

For hematologic work fix thin pieces of tissue in Helly fluid for 12-16 hr. and wash under running water for 24 hr.

After primary fixation dehydrate and embed in paraffin in the usual manner.

1. Cut sections 3-5μ thick.
2. Deparaffinize in 2 applications of xylol, 2 min. each.
3. Remove xylol by 2 applications of absolute alcohol or anhydrous isopropanol.
4. Treat with tincture of iodine for 5 min. to remove the bichloride of mercury precipitate.
5. Treat with 1% aqueous sodium thiosulfate for 5-10 min.
6. Wash thoroughly in tap water, followed by neutral distilled water.
7. Stain in freshly prepared dilute Giemsa stain for 16-24 hr. The dilution is prepared by adding 1 drop of stock Giemsa stain to each 1 ml. neutral distilled water. After the first hour replace the dilute Giemsa mixture with a fresh supply.
8. Wash in distilled water.
9. Dehydrate and differentiate in 9 parts acetone plus 1 part xylol; then in 5 parts acetone plus 5 parts xylol; and finally in 1 part acetone plus 9 parts xylol.
10. Clear in neutral xylol. Mount in gum damar.

At times better results are obtained by clearing the sections in carbolxylol or creosote first, followed by neutral xylol.

Nuclei of protozoa stain red; nuclei of cells and neutrophil granules, red violet; basophil granules, blue violet; cytoplasm of lymphocytes, blue; eosinophilic granules and red blood cells, pale red.

Giemsa stain of tissue—Schilling method, Gradwohl modification

1. Immerse the tissue in Susa fixative (p. 1642) for 5-48 hr., depending on the size.
2. Wash in tap water for 3-5 hr., changing the water frequently.
3. Place in 50% alcohol for ½ day, changing alcohol several times.
4. Transfer to 70% alcohol for ½ day, changing alcohol several times.
5. Transfer to 95% alcohol for ½ day, changing alcohol several times.
6. Place in absolute alcohol not longer than 1 hr. Larger blocks should be left longer than 1 hr.
7. Clear in pure xylol. The tissue must be transparent.
8. Place the tissue in a mixture of equal parts of paraffin and xylol at a temperature not to exceed 37°-40° C. overnight. Use embedding paraffin with a melting point of 56°-58° C. Tissuemat may be used. Keep the paraffin oven at a temperature 2° above the melting point of paraffin.
9. Place tissues in pure paraffin or Tissuemat in a paraffin oven for 4 hr. Transfer to fresh paraffin or Tissuemat in a paraffin oven for 2-4 hr. Embed in fresh paraffin or Tissuemat in the usual manner.
10. Cut sections about 5μ thick, mount on slides using Mayer albumin fixative, and allow to dry thoroughly.
11. All solutions for staining sections should be in staining jars.
12. Place the slides in xylol for 5 min. to remove the paraffin.
13. Carry through absolute alcohol, 1 min.; 95% alcohol, 1 min.; 80% alcohol, 1 min.; 70% alcohol, 1 min.
14. Place in iodine-alcohol (100 ml. 70% alcohol and 1 ml. tincture of iodine) for 1-2 hr. or until tissues assume a brown color. Omit this step for formalin-fixed sections.
15. Treat in 0.2% sodium thiosulfate not longer than 10-15 min. or until the brown color is removed.

16. Wash for 30 min. in a jar of tap water.
17. Wash for 30 min. in a jar of distilled water.
18. Dilute Giemsa stain by adding 1 drop of Giemsa stain to each ml. of neutral distilled water, shaking while adding each drop. Avoid shaking too vigorously.
19. Stain for 30 min. on a staining bridge, flooding the slide with dilute Giemsa stain.
20. Renew the stain at the end of the 30 min. staining period, diluting a fresh supply for the restaining process. Restain for 30 min.
21. Wash briefly in a jar of distilled water.
22. Place the slide under the low-power objective of a microscope and decolorize as follows: Use a weak solution of acetic acid made by adding 1 or 2 drops of 1% acetic acid to 50 ml. distilled water. Have at hand a jar of slightly alkaline water, made by adding 2 or 3 drops of 1% potassium carbonate to 100 ml. tap water.
23. Place the Giemsa-stained slide under the microscope.
24. Flood the slide with the weak acetic acid solution, and as soon as the pink color of the eosin is observed, place the slide in the jar containing slightly alkalinized water. The rapidity of this change is important, because decolorization will continue as long as the acid remains not neutralized on the tissue. Carry out dehydration in staining jars.
25. Place the slide in the following: solution 1 for about 5-10 sec.; then in solution 2 for about 5-10 sec.; and then in solution 3 for 10 sec. or more.

    Solution 1:

Xylol	5 parts
Acetone	95 parts

    Solutions 2 and 3:

Xylol	30 parts
Acetone	70 parts

26. Place in xylol. If the section does not become transparent, place again for a few seconds in solution 3 and then back in xylol. Continue this process until the section is clear; then leave in xylol for several minutes. Keep the acetone solutions tightly stoppered to prevent rapid evaporation and absorption of undue amount of moisture. Mount in gum damar.

STAINING AMEBAE IN TISSUE

Heidenhain iron hematoxylin

Heidenhain iron hematoxylin is a standard method to demonstrate the cysts of *Entamoeba histolytica,* chromatin, nuclear membrane, centrosomes, and various secretory products. It is applicable after any standard method of fixation. Zenker fluid and alcohol are preferred.

1. Cut very thin frozen, paraffin, or celloidin sections and mount on slides in the usual manner. Remove the precipitate from mercury bichloride-fixed sections by treatment with iodine and place them for 1-24 hr. in a staining dish containing 5% aqueous iron alum (5 gm. iron and ammonium alum dissolved in 100 ml. distilled water).
2. Wash in tap water.
3. Place in staining dish containing Heidenhain hematoxylin formula 2 (p. 1658) for 1-4 hr.
4. Wash in tap water.
5. Differentiate in 2% aqueous iron alum. Control the destaining under the microscope by taking the section out of iron alum solution, dipping it in water, and examining under low-power magnification. Repeat the process until the sections are suitably destained.
6. Wash in a large basin of tap water for several minutes until all traces of iron alum are removed from the section.
7. Dehydrate first in 95% alcohol and then in absolute alcohol.
8. Blot and clear in 2 changes of xylol.
9. Mount in gum damar.

The nuclei of cysts, amebae, the chromatin, and nuclear membrane stain blue.

Krajian elastic tissue stain for amebae in tissue

1. Prepare thin frozen, paraffin, or celloidin sections and mount on slides in the usual manner.
2. Stain in Krajian elastic tissue solution (p. 1658) for 5 min.
3. Wash in tap water.
4. Destain in 1% acid alcohol until the background begins to clear.
5. Wash in tap water.
6. Treat with 5% aqueous zinc sulfate for 2 min.
7. Rinse in tap water.
8. Counterstain for 2 min. in van Gieson solution (p. 1663).
9. Rinse in tap water and blot dry.
10. Differentiate and dehydrate with oil of origanum-absolute alcohol (equal parts) solution for 2 min.
11. Clear in carbolxylol for 2 min.; follow with pure xylol for 3 min.
12. Mount in gum damar.

The nucleoli of amebae stain brownish blue; cytoplasm, pink; connective tissue, dark red.

STAINING NEGRI BODIES IN TISSUE

To establish the diagnosis of rabies it is necessary to find Negri bodies in the nerve cells of the central nervous system. These are generally round or oval and stain deeply with fuchsin; their granules take a blue color. Refer to p. 882 for further information.

Method 1—Goodpasture carbolfuchsin and methylene blue method

1. Fix suspected brain tissue in Zenker fluid, wash, dehydrate, and embed in paraffin in the usual manner.
2. Cut very thin sections and mount on slides as usual. Carry through to water.
3. Remove the mercury precipitate by treating in tincture of iodine and then in 5% sodium hyposulfite.

4. Wash very thoroughly in tap water.
5. Stain in Goodpasture fuchsin solution formula 2 (p. 1661) for 10-30 min.
6. Wash off excess stain in running water.
7. Blot with fine filter paper.
8. Decolorize in 95% alcohol until the section becomes pink.
9. Wash in tap water and counterstain for 15-60 sec. with Loeffler methylene blue.
10. Wash in tap water.
11. Decolorize and at the same time dehydrate in absolute alcohol for a few seconds until the excess blue is removed.
12. Clear in 2 changes of pure xylol.
13. Mount in gum damar.

This method is useful for the demonstration of Negri bodies, bacteria, and rickettsiae. The staining solution is not stable. It will be convenient to keep the required alcoholic solution of fuchsin on hand, adding it to the phenol and aniline oil at the time of use.

Method 2—Negri body stain in tissue section

1. Embed Zenker-fixed brain tissue in paraffin in the usual manner, cut thin sections, and dry.
2. Remove paraffin with 2 applications of xylol, 2 min. each.
3. Wash with 2 applications of anhydrous isopropanol or absolute alcohol, 1 min. each.
4. Treat with tincture of iodine for 10 min. to remove the mercury.
5. Wash in isopropanol or 95% alcohol.
6. Wash thoroughly in water.
7. Stain in dilute fuchsin-methylene blue (p. 1661) for 7-10 min., gently steaming.
8. Rinse in tap water and blot.
9. Treat with anhydrous isopropanol or absolute alcohol for 15-25 sec.
10. Blot dry and immediately dip in and out in carbolxylol until the background is completely red. Control this step under low magnification of a microscope; if the nuclei are very deep blue, continue the carbolxylol treatment.
11. Clear in 2 changes of xylol, 2 min. each.
12. Mount in gum damar.

The stock staining solution may be kept for a long period. Shake thoroughly before diluting it.

The diluted solution does not keep longer than 15 min., and for this reason fresh solution should be prepared before use.

This is a highly satisfactory method to demonstrate Negri bodies in tissue sections.

Negri bodies in smear preparation—Frothingham

1. Choose small pieces of gray matter from the hippocampus or vermis of the cerebellum and place on a clean slide. Prepare smears and air-dry. Fix in absolute alcohol for a few seconds.
2. Stain smears in cold Frothingham fuchsin-methylene blue (p. 1661), dry, and examine under oil-immersion objective.

The Negri bodies are exceptionally brilliant red with blue granules.

SPIROCHETE STAINS

Among numerous similar organisms *Treponema pallidum* stands out as peculiar in its resistance to stains. Many methods have been devised to demonstrate it, in both the living and dead state.

Krajian 20 min. stain for Treponema pallidum in frozen sections

In 1939 Krajian described his excellent and practical method for demonstrating *Treponema pallidum* in biopsy material particularly designed to facilitate the diagnosis of primary syphilis. Gradwohl stated that it is the best and simplest method yet devised. It gives a clear-cut picture of the organism of syphilis, even in many instances in which dark-field examinations have failed to demonstrate it.

The dark-field method is positive in about 90% of cases in the first week of the lesion and in 50% in the fifth week. The morphology of the spirochetes found on the surface of mucosal lesions by dark-field and smear methods may be of less significance than those found deep in the tissues of biopsy sections because here their recognition is not affected by saprophytic organisms and free oxygen.

The 20 min. method of Krajian follows.[47, 48]

Reagents:

1. Primary solution.

Uranium nitrate	1 gm.
Formic acid, c.p., 85%	3 ml.
Glycerin, c.p.	5 ml.
Acetone, c.p.	10 ml.
Alcohol, 95%	10 ml.

2. Silver nitrate solution, 10%.
   (a) Dissolve 10 gm. silver nitrate, c.p., in enough distilled water to make 100 ml. solution. The 1% solution is made from this.
3. Silver nitrate solution, 1%.
   (a) The 10% silver nitrate solution will keep for months. Dilute it just before using by adding 3 ml. 10% silver nitrate to enough distilled water to make 30 ml. solution.
   (b) This solution may be used a maximum of 3 times during the course of the same day. Do not use it if it has a brownish cast.
4. Developing solution.

Hydroquinone	0.31 gm.
Sodium sulfite	0.1 gm.
Acetone	2.5 ml.
Formaldehyde, 40%	2.5 ml.
Pyridine	2.5 ml.

Saturated solution of gum mastic in 100% alcohol	2.5 ml.
Distilled water	15 ml.

(a) Dissolve the hydroquinone in formalin and acetone; then dissolve the sodium sulfite in this solution. Then add the other reagents and mix. Finally add the gum mastic. With the addition of the water the mixture assumes a smooth creamy appearance.

(b) This solution may be used a maximum of 3 times during a day's work but should preferably be used in small quantities and used only once. The stock solution, if kept out of the light, will remain good for at least 1 or 2 wk. Keep out of the cold to prevent separation of the gum.

5. Saturated alcoholic solution of gum mastic.
   (a) Saturate 25 gm. gum mastic with 35 ml. absolute alcohol. Shake several times a day for 3-5 days until clear. Allow to precipitate and use only the clear supernatant portion. Clarification can be hastened by centrifuging.

Procedure:

1. If the tissue has not been previously fixed in 10% formalin, boil a small piece of biopsy material in 10% formalin in a Pyrex test tube, agitating it for 1 min., or drop blocks of tissue about 5 mm. thick in 10% formalin at 67° C. and place in an oven at 67° C. for 10 min.
2. Freeze and cut immediately. Cut frozen sections about 7-10μ thick. Wash in distilled water.
3. Place the *primary solution* and the *developing solution* in small Stender dishes in a paraffin oven at 60° C. to keep them warm. Have 3 small Stender dishes containing distilled water. Place about 5-10 ml. 95% alcohol in another Stender dish.
4. In another Stender dish place 5 ml. 95% alcohol and add about 3 drops of saturated solution of gum mastic from the end of a small glass lifter.
5. The 1% silver nitrate solution should be in a 250 ml. Pyrex beaker.
6. Place the tissue in the primary solution at 56°-60° C. for 5 min. This can be kept in a paraffin oven to maintain its temperature.
7. Rinse in distilled water in a Stender dish. The tissue should open and float on the water.
8. Pick up the section with a glass lifter and, keeping it on the lifter, pass 3 times quickly but gently through the weak gum mastic mixture in a Stender dish.
9. Place the section in another Stender dish of distilled water. The section should open and float on the water.
10. Transfer the section to a wide-mouthed 250 ml. Pyrex beaker containing 30 ml. 1% silver nitrate. Put the beaker on an asbestos board over a low flame and under a 60-watt electric light, which should be about 4 ft. above the beaker. Heat the silver nitrate under the electric light until bubbles form, keeping the temperature between 70° and 73° C. for 7 min.
11. Without washing, carry individual sections from the silver nitrate to the warm developing solution, using the glass rod lifter. Alternately lift in and out of the solution, exposing every part of the section to the electric light. When replacing each time, take care that the section opens out on the developing solution. This cannot be accomplished unless the solution is quite warm. Repeat this alternate exposing and developing process about 6-8 times. The tissue turns brown.
12. Transfer to 95% alcohol in a Stender dish to remove excess gum mastic.
13. Transfer to another Stender dish containing fresh distilled water. The tissue should straighten out here. If it does not, replace for a few seconds in the alcohol and then place in the distilled water.
14. Place the tissue again in the **cooled** silver nitrate solution and expose to the electric light for an additional 10-30 sec. to cause the treponemes to assume a good black color.
15. Transfer to a large basin of distilled water. **Do not use tap water.**
16. Transfer to a glass slide. Dehydrate by dropping isopropanol over the sulface; blow gently at first and then blow a little harder to remove the water. Dehydrate in isopropanol about 30 sec.
17. Blot under several thicknesses of No. 1 Whatman filter paper, place isopropanol over the slide, and allow to dehydrate for at least 1 min. Again blot with Whatman No. 1 filter paper after draining off the excess isopropanol.
18. Dip once in thin celloidin (p. 1666). Wipe the bottom of the slide dry. Blow over the surface of the celloidin until it is thoroughly dry.
19. Place the slide on a staining dish and cover with isopropanol for 60 sec. to dehydrate.
20. Place in xylol for several minutes. Place in a second xylol to clear. Clean excess celloidin from around the tissue and mount with gum damar. Examine with the oil immersion objective.

Pointers: The spirochetes should stain black against a light brown background. If they are brown, either the second silvering process was not carried out properly or the developing solution was too cold. Or the developer might have separated, in which case make fresh developer.

When the gum mastic separates out of the developer, make a fresh solution. The primary solution keeps indefinitely.

If some of the spirochetes are brown and some are black, the sections were not spread properly in the developing solution. Be sure the solution is hot so that the tissues will open properly.

If some portions of the tissue do not show spirochetes and it is believed that they must be there, it may be due to improper fixation of the tissue. Unfixed portions of the section do not permit staining of the spirochetes.

When gum mastic is old or is in solution, it deteriorates.

If the lesion has been covered or treated with Mercurochrome or other chemicals the staining will be affected.

Dirty glassware will cause precipitates in the section. Clean with sulfuric acid-dichromate cleaning solution.

If the tissue suddenly shrinks when it is removed from the silver nitrate solution and placed in the water, this may be due to the fact that the water is not really distilled water, or the silver nitrate for the second silvering process was too hot and the temperature change too sudden. Sometimes going back to the hot developer and then through the other solutions will help unfold such a tissue. Or too weak a gum mastic solution was used.

It is good practice to stain a known positive section along with the specimen for diagnosis as a check on the solutions.

Never use a metal needle for removing tissues. Use the glass rod lifter.

This method is also applicable to paraffin sections, provided the period of staining in each step is doubled. It has been used successfully in our laboratories in demonstrating treponemes in primary sores where dark-field examinations have been negative. A small piece of tissue of the chancre, after injection of a few drops of Novocain solution, is snipped off with a pair of sharp scissors, and the above technic is carried out.

Krajian method for Treponema pallidum in smears

The silver stain for smears is capable of impregnating within a 15 min. period even the most resistant spirochetes and offers both convenient and permanent records.

Recognition of the morphologic characteristics of treponemes on the surface of mucosal lesions may be of less significance than recognition of them when deep in the tissue, but the finding of spirochetes with the morphologic characteristics of *T. pallidum* in smears from a suspicious lesion constitutes at least presumptive evidence of the syphilitic character of the lesion.

1. Moisten a swab in alcohol and roughly rub the lesion until it bleeds. Several minutes after all the bleeding stops, a clear serous exudate will appear on the abraded surface; from this fluid prepare and air-dry smears.
2. Flood the smears with warm (60° C.) primary solution (p. 1722) for 5 min. Dry in the air.
3. Wash in distilled water.
4. Treat with a weak solution of gum mastic for 2 min. (3 drops saturated alcoholic solution of gum mastic mixed with 7 ml. 95% alcohol).
5. Pour off the gum, blow the breath over the surface of the smear, and rinse in distilled water.
6. Set the slide on a metal stand or tripod, flood with 1% silver nitrate, and heat from under until bubbles arise, but do not boil the solution. Maintain the temperature for 3 min.; then repeat the silvering process once.
7. Pour off the silver nitrate; then without washing apply a thin coat of developing solution and leave the slide under an electric light for 2 min., warming gently with a flame. The developing solution is the same as that on p. 1722 except that 0.06 gm. sodium sulfite is used in place of 0.1 gm.
8. Wash with distilled water, dry in filter paper, and examine with an oil immersion objective.
9. For permanent mounts, dehydrate with absolute alcohol, blot, clear in xylol, mount in gum damar.

Clinical application of a rapid staining method for Treponema pallidum

A rapid staining technic for identifying *T. pallidum* is useful, for identification by the dark-field technic is a problem. Because of the usual location of the primary syphilitic lesions, saprophytic spiral forms, which are normal inhabitants, cause great confusion, particularly of the material from mouth and anal lesions. Many of those who have made a study of spirochetes have stated that they are unable to identify *T. pallidum* definitely in the presence of other spiral form organisms and therefore refuse to make a diagnosis under such circumstances.

Since the saprophytic types are surface inhabitants, the biopsy method completely avoids the confusion. It was stated by Noguchi and Stitt that *T. pallidum* is the only spirochete that retains its characteristic form after death; therefore, finding morphologically characteristic spiral form organisms deep in the tissue makes the diagnosis much simpler and reliable.

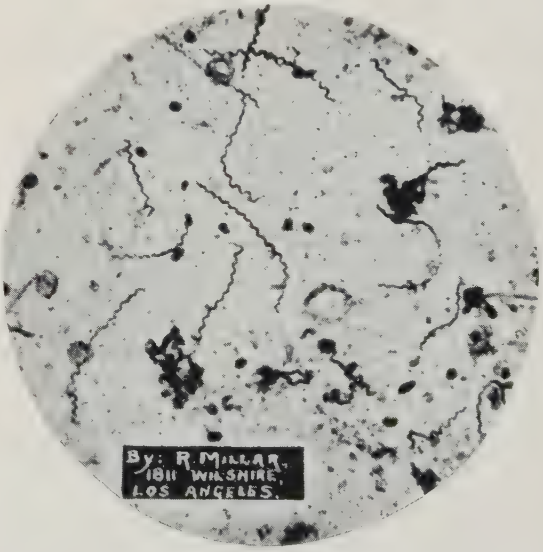


Fig. 568. *Treponema pallidum* in smear preparations. Krajian method.

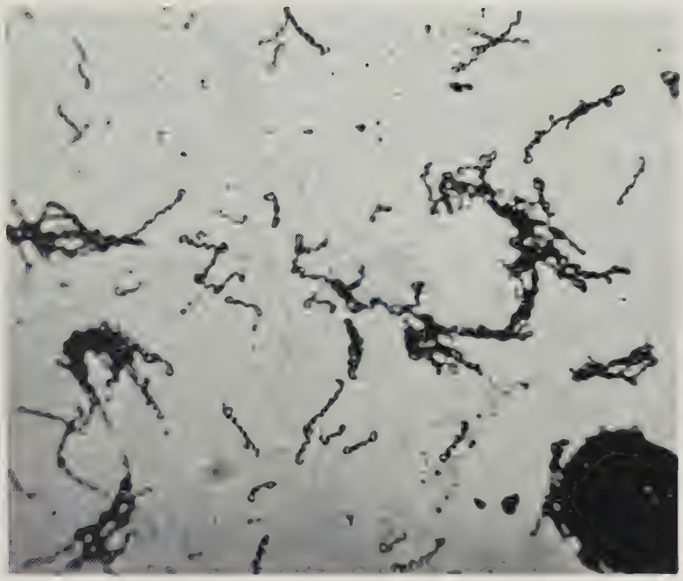


Fig. 569. *Leptospira icterohaemorrhagiae.* Smear preparation from culture. Krajian method. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

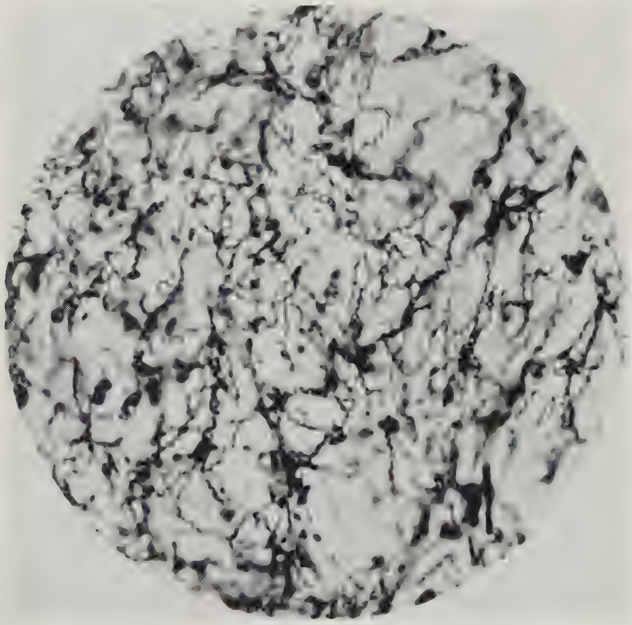


Fig. 570. *Treponema pallidum* in liver section from a case of congenital syphilis. Krajian method. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

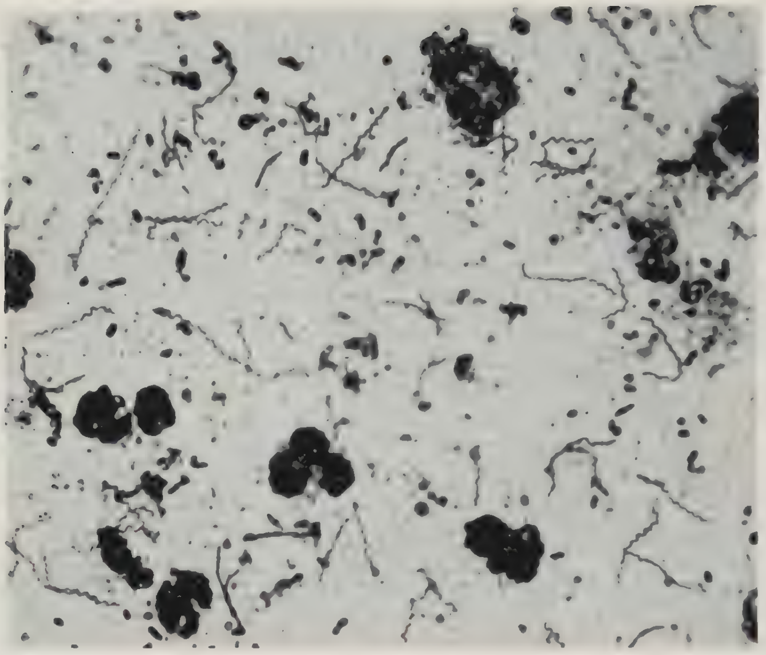


Fig. 571. *Treponema pallidum* in a section from primary lesion. Krajian method. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

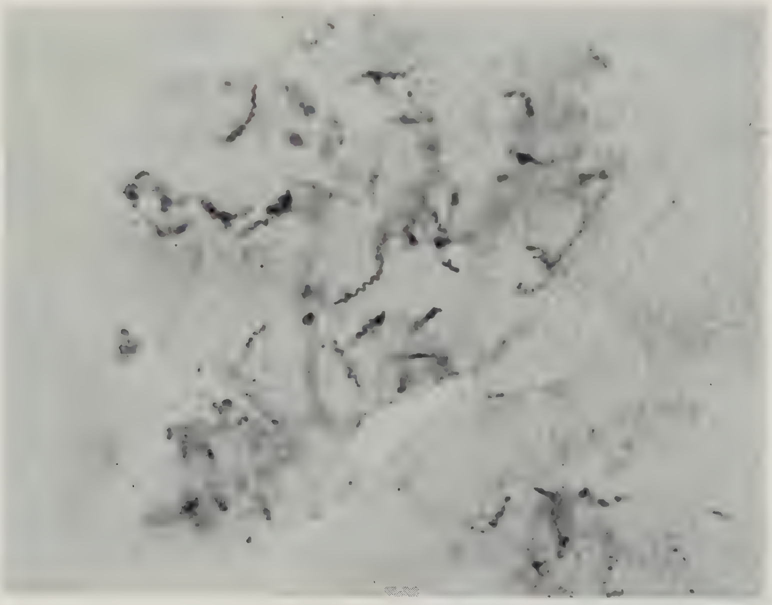


Fig. 572. *Treponema pallidum* in skin lesion. Secondary syphilis. Krajian method. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

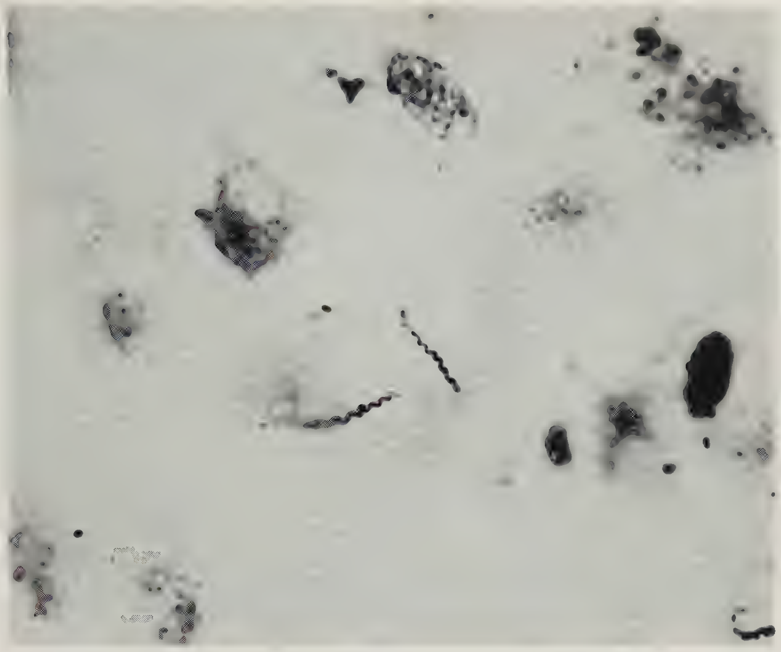


Fig. 573. *Treponema pallidum* in gumma of testicle. Tertiary lesion. Krajian method. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

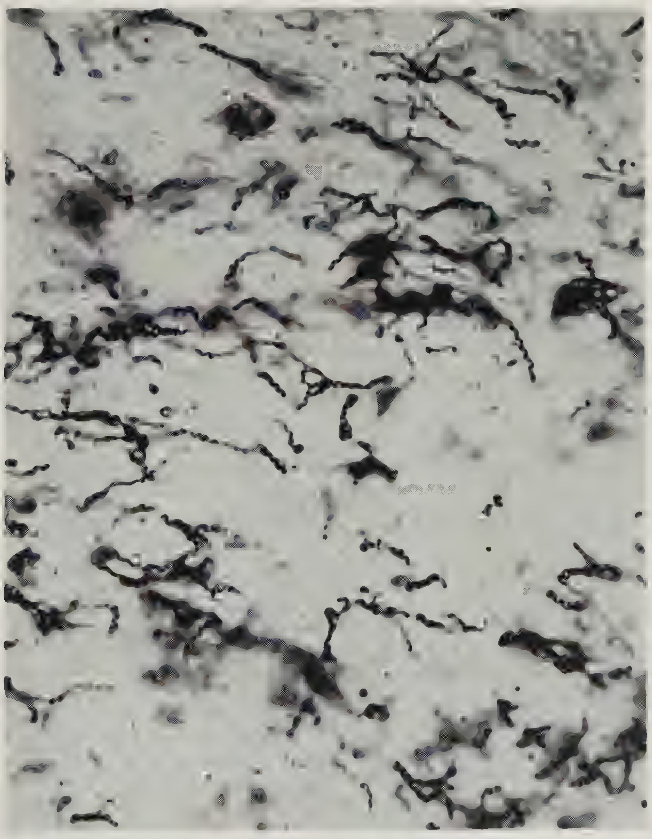


Fig. 574. Case of syphilitic aortitis. Note various forms of *Treponema pallidum,* especially broken and granular types. Krajian method. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

Levaditi-Manouelian stain for Treponema pallidum in tissue

1. Fix thin blocks of tissue in 10% formalin for 24 hr. or longer.
2. Harden in 95% alcohol for 24 hr.
3. Place the blocks in distilled water and wait until they sink to the bottom of the container.
4. Impregnate in 2% aqueous silver nitrate 3-6 days in an incubator at 37° C.
5. Rinse the block in distilled water for 2 min.
6. Reduce for 24 hr. at room temperature in the following solution, keeping the container in a dark place:

Pyrogallic acid	3 gm.
Formalin, 40%	5 ml.
Distilled water	100 ml.

7. Wash in distilled water for 15 min.
8. Dehydrate in graded alcohols, commencing with 70% alcohol and ending with absolute alcohol; then clear and embed in paraffin in the usual manner.
9. Cut thin paraffin sections, about 5μ thick.
10. Dissolve the paraffin in xylol and mount in gum damar.

Treponemes are jet black with a yellow background.

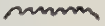
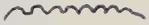
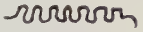
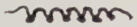
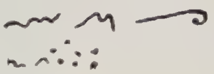
The Levaditi and Manouelian method is included for historical purposes.

Morphologic variations of Treponema pallidum

Morphologic variations of *T. pallidum* are best observed in tissue sections and smears. The typical form has regular, delicately curved, corkscrew-like, coil spring or knotty type appearance. Atypical forms are irregular in size and shape.

In tissue they are longer and thinner, especially when in large numbers (in very early lesions), when they are forced to struggle for existence. They become thicker and shorter when certain obstacles arise, such as slight administration of oxygen, or when in pus or exudate.

It has been stated that spirochetes have a quiescent stage during which they change morphology because of the knots, buds, wirelike, ringlike, granular, fragmented, sawlike, stretched, and other unusual forms. Most investigators believe that these atypical forms are part of the evolutionary cycle of the organism; others think that they are products of degeneration. These degenerative and atypical forms are generally seen in gummas where there is extensive destruction of the tissue; here can be observed many peculiar forms, small particles, or remnants of the destroyed organisms. These peculiar forms are generally observed in smears or tissue sections from experimental animals in which there has been a delay of several days in fixing them. They are also seen in unfixed syphilitic human organs. These findings suggest that the atypical forms are products of degeneration.

1. Tightly woven treponemes with tapering ends, as seen in congenital syphilis. 
2. Long, delicate forms, as seen in primary syphilitic lesions. 
3. Coil spring type, as seen in condylomas. 
4. Knotty type, as seen in umbilical cords. 
5. Atypical forms, broken and granular, as seen in tertiary lesions (gummas). 

STAINING FUNGI IN TISSUE

Actinomyces

It is generally thought that filaments seen in tissue in the actinomycoses, when stained by the Gram-Weigert method, retain the gentian violet. While examining biopsy sections from various skin lesions to demonstrate *T. pallidum* by the Krajian method, several cases were encountered which showed filaments of streptothrix. The same sections when stained by Gram-Weigert method gave negative results. It was concluded that some of these filaments belong to the gram-negative group. It is advantageous to use both methods in suspected cases.

Coccidioides and Blastomyces

Coccidioides and *Blastomyces* are easily stained and demonstrated by the routine hematoxylin-eosinol method. See Figs. 575 and 576.

STAINING PIGMENTS IN TISSUE

Pigments found normally or abnormally in the human body may be roughly divided into 3 groups according to their origin: (1) hematogenous pigments, (2) autogenous pigments, and (3) exogenous pigments.

Hematogenous pigments

From the standpoint of pathology, hemosiderin is the most important of the hematogenous pigments.

Hemoglobin occurs as yellowish brown granules and droplets. It is soluble in water and dilute alcohol but not in absolute alcohol.

Hematin occurs as a dark brown or bluish black pigment.

Hemofuscin occurs as light yellow granules. It does not give the iron reaction with potas-

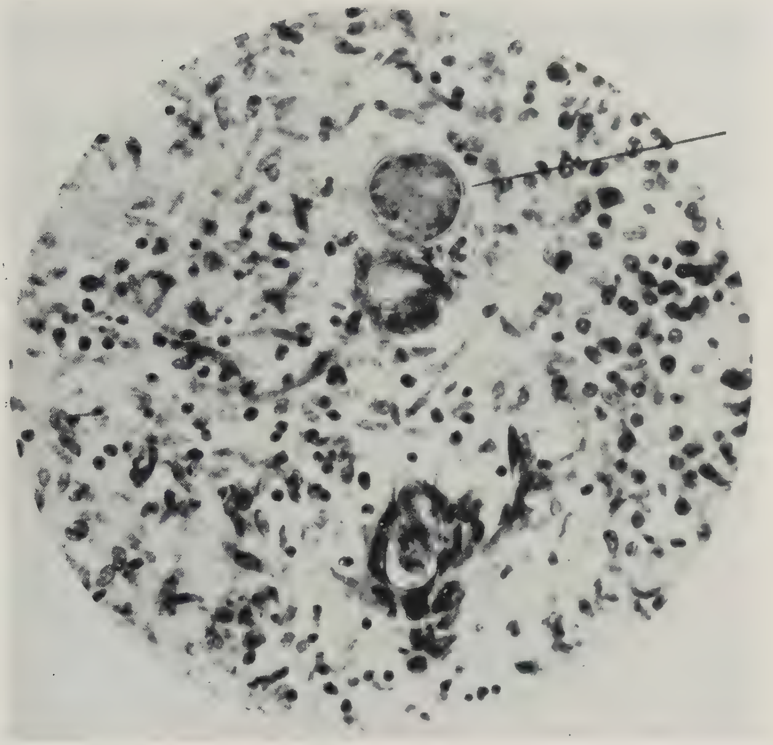


Fig. 575. *Coccidioides.* Hematoxylin-eosinol stain. Frozen section. Line leads to *Coccidioides.* (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

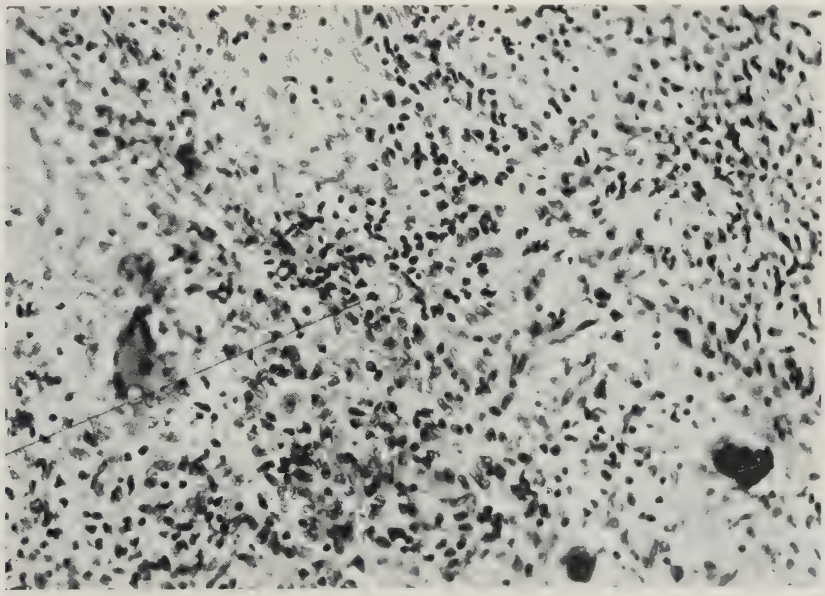


Fig. 576. *Blastomyces* in biopsy section. Hematoxylin-eosinol stain. Frozen section. Line leads to *Blastomyces.* (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

sium ferrocyanide, is not soluble in hydrogen peroxide, and is found in liver and other organs in hemochromatosis.

Hemozoin occurs as black granules formed by malarial parasites living in red blood cells; it can be distinguished from carbon by its solubility in concentrated sulfuric acid. Hemozoin pigments do not give iron reaction.

Hemosiderin occurs as bright yellowish brown granules and masses. It is not soluble in water, alcohol, ether, or alkalies but is soluble in acids. It does not stain with fat stains.

Hematoidin occurs as orange-colored or red rhombic plates or as radiating bunches of yellow needles. It contains no iron, is not soluble in water, alcohol, or ether, but dissolves in chloroform. It is found in old hemorrhages, especially in infarcts of the spleen and brain.

Bile pigments occur as yellowish granules and masses. They are not soluble in water, alcohol, or ether.

Mallory fuchsin stain for hemofuscin:

1. Fix tissues in Zenker fluid, alcohol, or formalin, and make paraffin, celloidin, or frozen sections. Mount on slides and carry through alcohols into water as usual.
2. Stain in Harris hematoxylin until the nuclei stand out sharply.
3. Wash in tap water.
4. Stain for 10-20 min. in Mallory basic fuchsin (p. 1661).
5. Wash in water.
6. Differentiate and dehydrate in 95% alcohol, followed by absolute alcohol.
7. Clear in xylol and mount in colophonium.

Iron reaction with potassium ferrocyanide:

1. Make frozen, paraffin, or celloidin sections in the usual manner and stain for 10-20 min. in the following mixture:

Potassium ferrocyanide, 2% aqueous, freshly prepared	1 part
Hydrochloric acid, 1%	3 parts

2. Best results are obtained if the fluid is warmed. Heat the mixture in a clean Pyrex test tube until beads of vapor form on the inner surface of the glass, about 70° C. Then place the sections in it or pour the mixture over them in a Stender dish. The reaction takes place within ½-2 min.
3. Wash thoroughly in several changes of distilled water. Fix the sections to the glass slide with thin celloidin (p. 1666).
4. Counterstain in eosin. Rinse in water.
5. Dehydrate completely with 2 changes of absolute alcohol.
6. Clear in pure xylol.
7. Mount in gum damar.
8. Clear celloidin sections in oil of origanum.
9. Use distilled water for the solutions and washings.

Nuclei and hemofuscin stain bright red; hemosiderin, blue.

Staining slices of gross specimens for iron pigments: The potassium ferocyanide and hydrochloric acid method may easily be applied to slices of fresh tissue for diagnostic purposes or after fixation. Attractive museum preparations may be made and preserved.

Fresh tissues: Cut thin slices of tissue from suspected iron-containing organs and place in the potassium ferrocyanide-hydrochloric acid mixture. If iron is present, the blue reaction takes place within 2-10 min.

Museum specimens:

1. Fix large slices of liver or other organs containing iron in Kaiserling solution 1 (p. 2068) for several days.
2. Wash thoroughly under running water overnight.
3. Preserve in 80% alcohol.
4. Cut into thin slices to obtain smooth surfaces.
5. Place the slices in freshly prepared potassium ferrocyanide solution (p. 1730) warmed to 70° C. The reaction takes place in 2-5 min.
6. Wash under running water for 24 hr.
7. Mount in 80% alcohol along with an unstained slice for contrast. Do not use Kaiserling solution 3 because it causes the blue color to diffuse.

Autogenous pigments

Autogenous pigments occur normally in hairs and in the deeper layers of the epidermis, most abundantly in persons of dark complexion. These pigments produced in the body are termed "melanin." Melanin is a dark brown substance of great coloring power which occurs in cells as brown or black granules. It contains no iron or fat and does not react with iron or fat stains. It is not soluble in ordinary reagents except in alkalies. It is stained black by the silver nitrate method.

Masson melanin stain:

1. Make frozen, paraffin, or celloidin sections in the usual manner and carry through the various solutions to water.
2. Wash the sections in slightly ammoniacal distilled water (1 drop ammonium hydroxide to 100 ml. distilled water) for 2 or 3 hr.
3. Transfer sections to Fontana liquid for 2-3 hr.

Fontana liquid:

- (a) Add ammonium hydroxide to 5% aqueous silver nitrate until the precipitate which is formed is just dissolved.
- (b) Then add 5% silver nitrate drop by drop until the liquid is opalescent.
- (c) Let the fluid clear by standing.

4. Wash in distilled water.
5. Place sections in toning solution for 10 min.

Toning solution:

(a)

Ammonium sulfocyanate, 2% aqueous	3 ml.
Sodium hyposulfite	3 gm.
Distilled water	100 ml.

(b) Gold chloride,
    1% aqueous . . . . . 100 ml.
  Mix equal parts of (a) and (b) just before use.

6. Wash in distilled water.
7. Wash for 1 min. in 5% sodium hyposulfite.
8. Wash in several changes of distilled water.
9. Dehydrate in 95% alcohol; then in absolute alcohol.
10. Clear in xylol and mount in gum damar.

Melanin pigments assume a black color.

Exogenous pigments

Various substances enter the human body and are deposited there as pigments, among which the most important are carotin, carbon, iron, and silver.

Carotin is an orange yellow pigment derived from carrots, squash, and certain other vegetables. It is easily soluble in fats and fat solvents. It is not stained with fat stains.

Carbon occurs commonly and abundantly in the lungs and bronchial lymph nodes. At times it is carried to the spleen and liver, where recognition is important. It must be distinguished from malarial pigment. The distinguishing characteristics are its extreme black color and its insolubility in concentrated sulfuric acid, in which all other pigments dissolve.

Silver appears brown or black; it is turned black by ammonium sulfide and is removed by a mixture of potassium ferrocyanide and sodium hyposulfite.

Arsenic pigments—microchemical detection of arsenic in tissue[49]:

1. Fix the tissue in 10% formalin for 24-48 hr.
2. Wash in running water for at least 6 hr.
3. Cut in small pieces not over 2 mm. thick.
4. Place pieces of the tissue in a 1 oz. ground glass-stoppered bottle and fill the bottle nearly full with fresh neutral hydrogen sulfide solution. Place a small amount of petroleum jelly on the stopper and insert tightly; then bind down with a tape or cord. Incubate the bottle and contents between 70° and 80° C. for 4 days. Staining qualities may be better preserved if the tissue is incubated at a lower temperature for a longer time, 56° C. for 6 days. Change the neutral hydrogen sulfide daily.
5. Wash under running water for 6-12 hr.
6. Dehydrate by successive passages through 50, 70, 80, 95%, and absolute alcohol, each containing about 10% ether.
7. Embed in celloidin or paraffin and cut sections about 5μ thick. Remove the embedding medium from the section on the slide in the usual manner.
8. Place sections for 20 min. in 10% alcoholic hydrochloric acid to dissolve all sulfides except the arsenic trisulfide. Unfortunately this also seriously impairs the staining qualities of the tissue.
9. Wash in 70% alcohol for 20 min. and stain with hematoxylin and eosin as for routine section preparation.
10. Differentiate with 95% alcohol.
11. Clear in oil of cloves. Remove the excess with xylol.
12. Mount in gum damar.

Krajian argentaffin cell stain of frozen sections:

1. Cut thin frozen sections of formalin-fixed tissue.
2. Place for 30 min. in 10% ammonium hydroxide at 56° C.
3. Impregnate for 2 hr. at 55° C. in **ammoniacal silver solution.**
   (a) To 15 ml. 10% aqueous silver nitrate add strong ammonium hydroxide (28%) drop by drop, shaking well, until the precipitate of silver oxide is just dissolved; then add a few drops of 10% silver nitrate until there is a persistent opalescence.
4. Rinse in distilled water.
5. Tone in 1:300 gold chloride for 5 min.
6. Treat for 5 min. in 5% sodium hyposulfite solution.
7. Wash in distilled water.
8. Transfer to glass slides in the usual manner.
9. Dehydrate with isopropanol or absolute alcohol.
10. Clear in xylol.
11. Mount in gum damar.

Argentaffin granules stain opaque black; nuclei, light brown.

Calcium salts: The term "calcification" is applied to infiltration of tissue with calcium phosphate and calcium carbonate. The salts appear under the microscope as small refractive granules which may be mistaken for fat. They are dissolved in 5% nitric or hydrochloric acid.

Silver method for calcium in tissue—von Kossa:

1. Fix tissues in neutral 10% formalin or alcohol and make frozen, paraffin, or celloidin sections.
2. Place sections in 1.5% aqueous silver nitrate for 30-60 min.
3. Wash in distilled water.
4. Counterstain in safranin.
5. Dehydrate in absolute alcohol.
6. Clear in xylol and mount in gum damar.

Calcium salts appear as opaque black areas in the section.

Purpurin method for calcium in tissue:

1. Fix tissues in 10% neutral formalin or alcohol and make paraffin, celloidin, or frozen sections.
2. Stain in saturated alcoholic solution of purpurin for 5-10 min.
3. Rinse in 0.75% sodium chloride for 3-5 min.
4. Differentiate in 70% alcohol until the stain ceases to wash out of the section.
5. Dehydrate rapidly in absolute alcohol.

6. Clear in oil of origanum and mount in gum damar.

Calcium salts stain red.

CHROMATIN STAIN

1. Fix tissues preferably in mercuric chloride-acetic acid. Embed in paraffin and cut sections in the usual manner.
2. Stain in **Unna pyronine methyl green** stain for 10-30 min.

Methyl green (pure)	0.15 gm.
Pyronine	0.25 gm.
Alcohol, 95%	2.5 ml.
Glycerin	20 ml.
Phenol, 0.5% aqueous	100 ml.

The solution keeps indefinitely.

3. Wash in distilled water.
4. Blot in filter paper.
5. Dehydrate rapidly in absolute alcohol.
6. Clear in xylol and mount in gum damar.

Chromatin and nucleoli are fairly selectively stained green. Cytoplasm and bacteria stain red. The stain is not permanent.

CHROMAFFIN TISSUE STAINS

Wiesel method for chromaffin

1. Fix tissues for 1-4 days in the following fluid:

Potassium dichromate, 5% aqueous	10 ml.
Formalin, 10%	20 ml.
Distilled water	20 ml.

2. Treat with 5% potassium dichromate for 1-2 days.
3. Wash overnight under running water.
4. Dehydrate, clear, and embed in paraffin.
5. Stain sections for 20 min. in 1% aqueous toluidine blue or wasserblau.
6. Wash in water for 5 min.
7. Stain for 20 min. in 1% aqueous safranin. This may be omitted if desired.
8. Treat with 95% alcohol until the sections turn blue.
9. Dehydrate in absolute alcohol.
10. Clear in xylol. Mount in gum damar.

Chromaffin cells stain green; nuclei, red; cytoplasm of adrenal cortical cells, light blue.

Better results are obtained by omitting the safranin.

Giemsa method for chromaffin

1. Fix tissues in formol-dichromate mixtures as in the Wiesel method.
2. Wash in running water.
3. Make frozen sections (preferable) or embed in paraffin and cut sections.
4. Stain sections for 3-24 hr. in dilute Giemsa stain (1 drop Giemsa stain for every ml. neutral distilled water).
5. Wash in water.
6. Blot in fine paper.
7. Dehydrate with acid-free acetone and control differentiation under a microscope.
8. Clear in xylol and mount in thick cedarwood oil. Mounting in gum damar or balsam causes the sections to fade.

Medullary cells stain bright red to violet; cortical cells, blue to blue violet; red blood cells, pink.

PANCREATIC ISLET CELL STAINS

Lane-Bensley method

To distinguish between the α and β cells of the pancreatic islet tissue, Lane devised the following technic.

1. Fix small fragments of pancreatic tissue for 3-4 hr. in the following fluid:

Potassium dichromate	2.5 gm.
Mercuric chloride	5 gm.
Distilled water	100 ml.

2. Dehydrate, clear, and embed in paraffin in the usual manner.
3. Cut sections 3-5μ thick. Carry through solutions to water.
4. Stain for 24 hr. in Bensley neutral gentian violet (p. 1661).
5. Blot section in filter paper.
6. Dehydrate and partly differentiate by a few seconds' treatment with absolute alcohol.
7. Blot rapidly.
8. Flood the slide with oil of cloves and control the differentiation under a microscope until acinus cells show purple zymogen granules and their ground cytoplasm is brownish yellow.
9. Clear sections in pure xylol.
10. Mount in gum damar.

α **cells:** Cytoplasm barely stained; nuclei, pale purple.

β **cells:** Cytoplasmic granules, brilliant purple; nuclei, light purple.

Acinus cells: Zymogen granules, purple; ground cytoplasm, yellow.

No acetic acid should be added to the dichromate solution since it dissolves the granules of islet cells.

Bensley acid fuchsin methyl green stain

1. Fix tissues for 24 hr. in the following solution:

Osmic acid, 4% aqueous	2 ml.
Potassium dichromate, 2.5% aqueous	8 ml.
Glacial acetic acid	1 drop

2. Embed in paraffin in the usual manner and cut sections 5μ thick. Zenker-fixed tissues can also be stained by this method.
3. Free the sections from paraffin by toluol; then carry through absolute alcohol to water.
4. Treat with 1% aqueous potassium permanganate for 1 min.
5. Treat with 5% oxalic acid for 1 min.
6. Wash thoroughly in water.
7. Stain for 5 min. in aniline acid fuchsin solution (p. 1661) previously warmed to 60° C.
8. Wash thoroughly in water.

9. Dip for a few seconds in 1% aqueous methyl green.
10. Wash in water.
11. Dehydrate rapidly in absolute alcohol.
12. Clear in toluol and mount in gum damar.

Acinus cells stain green with green nuclei; zymogen granules, red; basal filaments and mitochondria, red; granules of α cells, deep red; granules of β cells, green.

Goodpasture acid polychrome methylene blue stain

This stain is used to differentiate α and β cells of islet tissue.

1. Fix thin pieces of fresh pancreas for 24 hr. in the following:
   Formalin, neutral . . . . . 10 ml.
   Zenker fluid without acetic acid 90 ml.
2. Wash under running water overnight.
3. Dehydrate in graded alcohols and embed in paraffin. Cut very thin sections, 3-5μ thick. Dissolve the paraffin in xylol; wash in alcohol and then in water.
4. Treat in 1% aqueous potassium permanganate for 1 min.
5. Treat in 5% aqueous oxalic acid for 1 min.
6. Wash thoroughly in tap water.
7. Stain in aqueous solution containing 1% eosin and 1% potassium dichromate for 1-5 min.
8. Wash rapidly in tap water.
9. Stain in acid polychrome methylene blue (p. 1660) for 1-5 min.; then wash rapidly in tap water.
10. Differentiate and dehydrate rapidly in 95% alcohol and then in absolute alcohol.
11. Clear in xylol and mount in gum damar.

Zymogen granules stain deep purple. Cytoplasm is light blue; nuclei, light purple; α granules bright red; β granules, dark blue.

Krajian method for zymogen granules

1. Prepare thin paraffin sections of Zenker- or formalin-fixed tissue.
2. Deparaffinize with 2 applications of xylol.
3. Remove xylol by anhydrous isopropanol or absolute ethyl alcohol, 3 applications.
4. Remove any mercury precipitate by Gram's iodine for 5 min. (if Zenker-fixed).
5. Differentiate in 5% strontium nitrate for 5 min.
6. Stain in **1% aqueous azocarmine** for 10 min.:
   Azocarmine . . . . . . 1 gm.
   Distilled water . . . . . 100 ml.
   Heat the solution for 20 min.
   Filter after cooling and acidify with 1 ml. glacial acetic acid.
7. Wash in tap water and place in 5% strontium nitrate for 5 min.
8. Wash in tap water and stain with **aniline blue–orange G** for 5 min.:
   Aniline blue . . . . . . 0.5 gm.
   Orange G . . . . . . 2 gm.
   Acetic acid . . . . . . 8 ml.
   Distilled water . . . . . 100 ml.
   Heat the mixture for several minutes, cool, and filter.
9. Wash in tap water and differentiate with 5% strontium nitrate for 3 min.
10. Wash and dehydrate 3 times with isopropanol or absolute alcohol.
11. Blot dry and partially clear with creosote-xylol for 5 min.
12. Pass through 2 changes of xylol, 3 min. each, and mount in Permount.

Zymogen granules are distinct bluish purple; reticulum and collagen fibers, brilliant blue; background, red. If formol-fixed tissue is used, mordant the section with thorium ferric for 10 min. after removal of the mercury.

PLASMA CELL STAIN

Unna method

Plasma cells are abundant in subacute and chronic infectious diseases.

1. Fix tissues in absolute alcohol. Fair results are obtained from tissues fixed in mercuric chloride, mercuric chloride-formol, or formalin.
2. Make paraffin or celloidin sections in the usual manner and carry through hydrating solutions to water.
3. Stain for 20-40 min. in Unna pyronine methyl green (p. 1660).
4. Wash in tap water.
5. Blot in filter paper.
6. Dehydrate very rapidly in absolute alcohol.
7. Clear in xylol.
8. Mount in gum damar.

Cytoplasm of plasma cells stains red; nuclei, bluish green. Lymphocytes also stain bluish green but are easily distinguished morphologically by their nuclei.

MAST CELL STAIN

Unna method

1. Fix tissues in absolute alcohol and make thin paraffin or celloidin sections. Carry through to water.
2. Stain in Unna polychrome methylene blue for 2 hr. in a paraffin oven or overnight at room temperature.
3. Wash in distilled water.
4. Dehydrate in 2 changes of 95% ethyl alcohol and blot.
5. Clear in oil of origanum for 5 min.
6. Treat with neutral xylol.
7. Mount in gum damar.

Mast cell granules stain red; nuclei, blue.

MITOTIC FIGURE STAIN

Mitotic figures are brought out sharply when tissue is perfectly fresh, that is, immediately after removal from a living organism. The details of mitotic figures are not so perfect in tissues removed at autopsy.

Tissue should be cut into very thin slices, not over 2 or 3 mm. thick, and fixed in a suitable fluid. Alcohol and Zenker fluid are preferable.

Staining with alum hematoxylin or safranin gives satisfactory results, but phosphotungstic acid-hematoxylin is highly recommended.

Mallory phosphotungstic acid-hematoxylin

1. Fix tissues in Zenker fluid for 16-24 hr.
2. Wash under running water overnight.
3. Dehydrate in graded alcohols, beginning with 70% and finally in absolute alcohol.
4. Embed in paraffin or celloidin, and cut sections. Carry through to water.
5. Treat with Lugol iodine solution for 5-10 min. to remove mercury precipitate.
6. Wash in 2 changes of 95% alcohol to remove every trace of iodine.
7. Wash in distilled water.
8. Treat in 0.25% aqueous potassium permanganate for 5-10 min.
9. Wash in tap water.
10. Treat in 5% aqueous oxalic acid for 5 min.
11. Wash thoroughly in several changes of tap water.
12. Stain in phosphotungstic acid-hematoxylin (p. 1658) for 24 hr.
13. Transfer directly to 95% alcohol, follow with absolute alcohol, and dehydrate quickly.
14. Clear in xylol (filter paper blotting method for celloidin sections).
15. Mount in gum damar.

Mitotic figures stain blue, as do the neuroglia, fibroglia, myoglia fibrils, and fibrin.

OXIDASE GRANULE STAIN

The following method is said to be useful for differentiating the granulocytic elements from the lymphocytic series of leukocytes; the myelogenous elements give a positive reaction, whereas the lymphocytes give a negative reaction.

Graham alpha-naphthol-pyronine method

The material must be formol-fixed, and freshly cut frozen sections must be used. After standing for 24-48 hr. in water, the granules may fail to react.

1. Stain sections lightly in Harris hematoxylin. The solution must not be too acid.
2. Wash in water and then for about 5 min. in saturated aqueous lithium carbonate. Return to water for a few minutes.
3. Stain for 10 min. in **alpha-naphthol-pyronine** staining solution:

Alpha-naphthol (Merck recrystallized or reagent grade)	1 gm.
Alcohol, 40%	100 ml.
Hydrogen peroxide	0.2 ml.

To 2 ml. of this solution add immediately before use 1 drop of 2% aqueous pyronine.

4. Wash in water; then place for 15-20 min. in saturated aqueous lithium carbonate. Wash thoroughly in several changes of water.
5. Differentiate and dehydrate in 80% alcohol, followed by 95% alcohol. Transfer to a slide and clear with xylol by the blotting method.
6. Mount in gum damar.

The oxidase granules stain intensely red; nuclei, greenish blue to blue.

HISTOPATHOLOGIC METHODS FOR NERVOUS SYSTEM TISSUE

NUCLEAR STAINS

Hematoxylin-eosin stain

Refer to pp. 1666, 1668, and 1691.

Ethyl violet-orange G stain

Stain:

Ethyl violet	1 gm.
Orange G	0.5 gm.
Distilled water	100 ml.

1. Stir to mix thoroughly.
2. Place in or on an oven for 12-24 hr. to precipitate.
3. Decant the supernatant fluid.
4. Wash the precipitate several times with distilled water.
5. Place in or on an oven to dry.
6. Dissolve in absolute alcohol (saturate).
7. For use add 1 part of the alcoholic solution to 3 parts of 20% alcohol.

Procedure:

1. Fix tissues in Zenker fluid and make paraffin sections in the usual manner. Formol-fixed paraffin sections can also be used if mordanted in Zenker fluid for 3 days. Carry through the hydrating solutions to water.
2. Fix in 3% potassium dichromate.
3. Treat in Lugol solution for 5 min.
4. Wash in water.
5. Stain in neutral ethyl violet-orange G for 12-24 hr.
6. Blot sections and wash in anhydrous acetone.
7. Dip in toluol and flood slide with pure clove oil.
8. Differentiate in a mixture of 3 parts of clove oil and 1 part of 95% alcohol.
9. Rinse in clove oil and wash in toluol.
10. Blot and clear in 2 changes of pure xylol.
11. Mount in gum damar.

Schultze-Stohr nerve cell stains for ganglion cells

1. Fix tissues in 10% formalin for 48 hr. or longer.

2. Cut frozen sections 30μ thick and place in distilled water.
3. Place in a solution consisting of 0.5 part of N/1 sodium hydroxide (4%) in 50 parts of distilled water for 24 hr. to remove granular precipitate.
4. Wash in a large quantity of distilled water for 1 hr., changing the water several times.
5. Place in 0.5% aqueous silver nitrate for 16 hr. or longer. The solution must remain clear when sections are placed in it.
6. Reduce in **hydroquinone solution,** made as follows:

Hydroquinone	2.5 gm.
Distilled water	100 ml.
Formalin	5 ml.

   The stock solution keeps for 3 mo. in a dark place. For reducing sections add 20 parts of distilled water to 1 part of stock solution.

7. Watch the reduction process under the microscope.
8. Wash quickly in distilled water and blot.
9. Dehydrate in several changes of absolute alcohol.
10. Clear in carbolxylol for 5 min.
11. Wash in 2 changes of pure xylol.
12. Mount in gum damar.

Cell bodies appear brown or black; dendrites, deep black.

Schultze-Stohr stain for brain tissue

1. Cut frozen sections 30μ thick and place in distilled water.
2. Place in sodium hydroxide solution for 24 hr. (6 parts of N/1 NaOH to 50 parts of distilled water).
3. Wash in distilled water for 1 hr. changing water frequently.
4. Place in 2% silver nitrate for 16 hr. or longer. There must be no clouding of the solution.
5. Reduce in hydroquinone solution (see above) for a few seconds and examine under the low power of a microscope. When the solution becomes cloudy, use fresh solution.
6. Wash in 2 changes of distilled water.
7. Dehydrate in 3 changes of absolute alcohol.
8. Clear in carbolxylol and then in 2 changes of xylol.
9. Mount in gum damar.

Stain for basal ganglia

1. Cut frozen sections 30μ thick and place them in sodium hydroxide solution (10 parts of N/1 NaOH to 50 parts of distilled water).
2. Wash in distilled water for 1 hr., changing the water frequently.
3. Place in 10% aqueous silver nitrate for 16-24 hr.
4. Reduce in hydroquinone solution (see above) for a few seconds. Examine under a microscope.
5. Wash in distilled water.
6. Dehydrate in 3 changes of absolute alcohol.
7. Clear in carbolxylol and then in 2 changes of pure xylol.
8. Mount in gum damar.

Stain for cerebellum

1. Cut frozen sections 30μ thick and place them in sodium hydroxide solution for 24 hr. (2 parts of N/1 NaOH to 50 parts of distilled water).
2. Wash in distilled water for 1 hr., changing the water frequently.
3. Place sections in 0.25% aqueous silver nitrate for 16-24 hr.
4. Reduce in hydroquinone solution (see above) for a few seconds, examining sections under a microscope.
5. Wash in distilled water.
6. Dehydrate in 3 changes of absolute alcohol.
7. Clear in carbolxylol and then in 2 changes of pure xylol.
8. Mount in gum damar.

Stain for medulla, spinal cord, and sympathetic ganglia

1. Cut frozen sections 30μ thick and place in sodium hydroxide solution for 24 hr. (10 parts of N/1 NaOH to 50 parts of distilled water).
2. Wash in distilled water for 4 hr., changing the water frequently.
3. Place sections in 10% aqueous silver nitrate for 16-24 hr.
4. Reduce in hydroquinone solution (see above) a few seconds, examining under a microscope.
5. Wash in distilled water.
6. Dehydrate in 3 changes of absolute alcohol.
7. Clear in carbolxylol and then in 2 changes of pure xylol.
8. Mount in gum damar.

Stain for glia

1. Cut frozen sections 30μ thick and place them in sodium hydroxide solution for 24 hr. (0.5 part of N/1 NaOH to 50 parts of distilled water).
2. Wash in distilled water for 48 hr., changing the water frequently.
3. Place sections in 0.5% aqueous silver nitrate for 16-24 hr.
4. Reduce in hydroquinone solution (see above) for a few seconds, examining sections under a microscope.
5. Wash in distilled water.
6. Dehydrate in 3 changes of absolute alcohol.
7. Clear in carbolxylol and then in 2 changes of pure xylol.
8. Mount in gum damar.

Stain for peripheral nerves

1. Cut frozen sections 30μ thick and place in sodium hydroxide solution for 24 hr. (10 parts of N/1 NaOH in 50 parts of distilled water).
2. Wash in distilled water for 1-2 hr., changing frequently.
3. Place in 10% aqueous silver nitrate for 24 hr.
4. Reduce in hydroquinone solution (see above). Since the reduction is very rapid and difficult to control, dilute the stock solution 100 times with distilled water and watch the process under the microscope.

5. Wash in distilled water.
6. Dehydrate in 3 changes of absolute alcohol.
7. Clear in carbolxylol and then in 2 changes of pure xylol.
8. Mount in gum damar.

When the nerve begins to appear during reduction, the section should at once be put into distilled water. If kept too long in the reduction fluid, the rest of the section stains the same dark color. If kept too short a time, the nerves cannot be identified; they stain incompletely or not at all. After the sections have been taken out of the reducing fluid and placed in distilled water, they cannot be returned to any fluid previously used. Thus sections should be taken out frequently from the reducing fluid and examined under a microscope to find the nerves.

NISSL GRANULE STAINS

Nissl's original method is long and complicated. The following methods may be used advantageously to demonstrate Nissl granules.

Toluidine blue stain for Nissl granules

1. Fix very thin blocks of tissue in 95% alcohol for 2-4 days.
2. Make thin paraffin or celloidin sections in the usual manner and hydrate in the usual manner.
3. Stain in 1% aqueous toluidine blue overnight at room temperature or 2 hr. in an oven at 55° C.
4. Rinse rapidly in water.
5. Dehydrate for 1 min. in 95% alcohol.
6. Differentiate in the following solution, controlling the extraction of stain under a microscope:

Creosote, pure	5 ml.
Cajuput oil	4 ml.
Xylol	5 ml.
Absolute alcohol	15 ml.

7. Rinse in absolute alcohol.
8. Clear in 2 changes of xylol.
9. Mount in gum damar.

Nissl granules stain blue against an unstained cytoplasmic background; nuclei remain nearly colorless.

Cyanin stain for Nissl granules

1. Cut frozen sections 5-10μ thick.
2. Transfer to slide.
3. Dehydrate and fix with celloidin in the usual manner for frozen sections.
4. Place in the following stain for 24-36 hr.:

Chrome alum	10 gm.
Distilled water	200 ml.

Mix and heat with 0.3 gm. cyanin.

Boil 20 min. Make up to original volume with distilled water. This keeps indefinitely. It should be filtered just before use.

5. Rinse in water.
6. Differentiate in 95% alcohol. Change 4 or 5 times.
7. Dehydrate rapidly in absolute alcohol.
8. Clear in xylol.
9. Mount in gum damar.

Cresyl echt violet stain for Nissl granules

1. Make thin frozen or paraffin sections in the usual manner.
2. Stain in 1% aqueous solution of cresyl echt violet (filtered) for 36 hr. at room temperature.
3. Wash in distilled water.
4. Differentiate in 95% alcohol until the background is white or nearly so.
5. Complete the dehydration in 3 changes of absolute alcohol.
6. Clear in 4 changes of pure xylol.
7. Mount in gum damar.

NERVE FIBER STAINS

Bielschowsky silver stain

Ammoniacal silver solution:

Silver nitrate, 10%	30 ml.
Sodium hydroxide, 40%	40 drops

1. A precipitate forms. Wash the precipitate a dozen times with distilled water, using approximately 1 L. water.
2. Add 50 ml. distilled water to the washed precipitate, and then add ammonium hydroxide drop by drop until the precipitate dissolves—avoid excess.
3. Dilute to 150 ml. with distilled water. Store in the dark.

Procedure:

1. Fix tissues in 15% formalin for 48 hr. or longer.
2. Wash several hours under running water.
3. Cut frozen sections about 10-15μ thick.
4. Wash thoroughly in distilled water.
5. Place in pyridine for 24-48 hr.
6. Wash in many changes of distilled water until the pyridine is completely eliminated.
7. Transfer to 3% silver nitrate for 24 hr. in a dark place at room temperature.
8. Take the sections one by one from the silver nitrate and wash quickly in distilled water.
9. Transfer to ammoniacal silver solution for about 10 min. until the sections become yellowish brown.
10. Wash quickly in distilled water.
11. Reduce in 20% aqueous formalin. If working with a number of sections, it is advisable to retransfer them to a fresh bath of 20% formalin. Reduction takes place at the end of 30 min. or less.
12. Wash in distilled water.
13. Tone in 2% gold chloride. Acidify the gold bath slightly with acetic acid to obtain a faintly purple background, or neutralize with a few drops of diluted sodium or lithium carbonate solution for a grayish background. A slightly acid solution of chloroplatinic acid may be used instead of gold chloride.
14. Wash in distilled water.
15. Treat in 5% sodium hyposulfite solution.
16. Wash in tap water.

17. Dehydrate completely in 3 changes of absolute alcohol.
18. Clear in carbolxylol 5 min.
19. Wash in 2 changes of pure xylol, and mount in gum damar.

Neurofibrils stain grayish black.

Cajal nerve fiber stain

This is an impregnation method for unmyelinated fibers in formalin-fixed tissues. Cajal nerve fiber stain is absolutely constant and is especially applicable to cerebellum.

1. Fix pieces of nerve tissue, especially cerebellum, in 15% formalin for 2 wk.
2. Cut frozen sections 15-20μ thick.
3. Place in 10% formalin. Wash rapidly in 2 changes of distilled water.
4. Place immediately in a freshly prepared mixture of 10 ml. 2% silver nitrate solution and 5 drops of pure pyridine.
5. Leave in the silver solution for 3-4 hr. if the

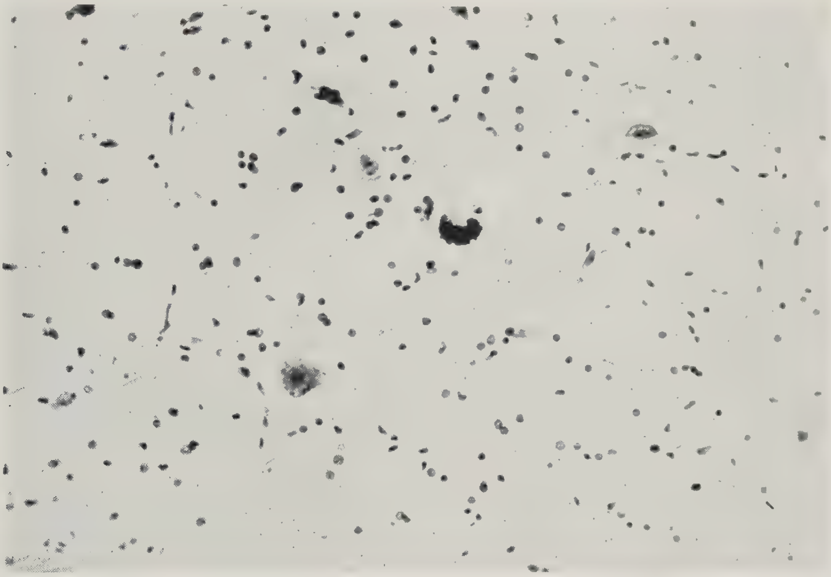


Fig. 577. Nerve cell stain. Cyanin method. Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

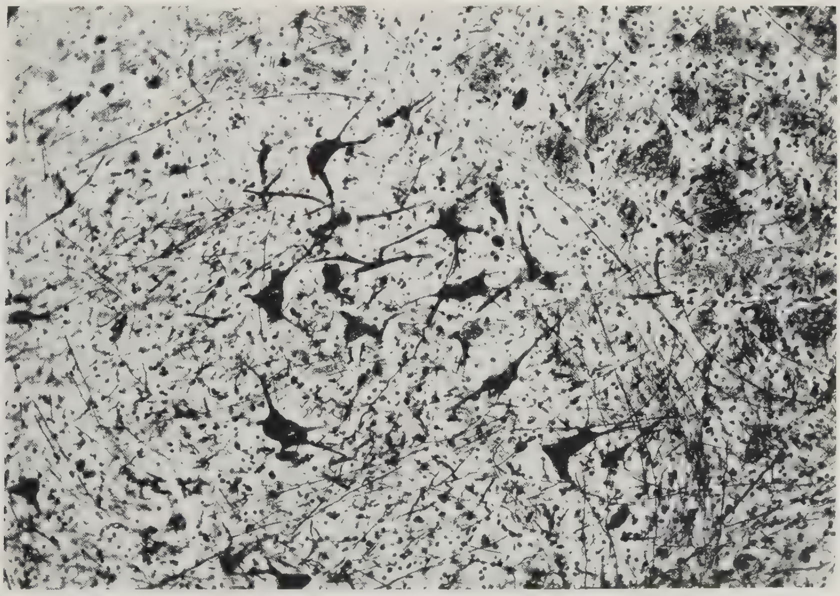


Fig. 578. Anterior horn cells of spinal cord. Reduced silver method (Cajal). Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic. St. Louis, 1952, The C. V. Mosby Co.)

tissues are fresh. Sections of old material should be left 20-40 hr. or until they are a tobacco color. Keep them in a dark place. If time is a factor, heat the solution for a few minutes.
6. Place in 10 ml. 95% alcohol for 1 min. If the impregnation is not intense (dark brown), add 2 or 3 drops of 2% silver nitrate solution to the alcohol bath.
7. Place sections without washing in the following **reducing solution:**

Hydroquinone, pure	0.2 gm.
Formalin (Merck)	30 ml.
Distilled water	70 ml.

Leave in this solution about 20 min.
8. Wash in distilled water.
9. Dehydrate in 3 changes of absolute alcohol.
10. Clear in carbolxylol for 5 min. Wash in 2 changes of pure xylol and mount in gum damar.

Unmyelinated fibers and chromatin stain black. If the sections are left in 95% alcohol for 6 hr. or longer before being placed in the reducing solution, the medullated fibers are well impregnated, but the cells remain unstained.

Only 1 section at a time should be placed in the alcohol bath.

NERVE ENDINGS AND PLEXUSES

Gold chloride method

Impregnation with gold chloride, when successful, produces a good picture of nerve endings and nerve plexuses.

1. Cut thin slices of fresh tissue.
2. Wash and place in physiologic saline and then in filtered lemon juice until they become clear. This requires 5-10 min.
3. Rinse with distilled water and place in 1% gold chloride (about 10 times their volume) in the dark for 30-40 min.
4. Rinse with distilled water and place in a mixture of 1 part of formic acid in 3 parts of distilled water. Keep in the dark for 24-48 hr., during which time the gold is reduced.
5. Wash in many changes of distilled water.
6. Tease apart the tissue block and mount in glycerin jelly (see Kaiser formula, p. 1655) or embed in paraffin in the usual manner, section, and mount in gum damar.

Nerve endings and plexuses stain violet to black.

MITOCHONDRIA STAIN

Bailey-Davis method

1. Fix tissues in Regaud fluid or 10% formalin and mordant 7-10 days in 3% potassium dichromate.
2. Wash, dehydrate, and embed in paraffin in the usual manner.
3. Cut sections 3-5μ thick. Carry thorough xylol, alcohols, to distilled water.
4. Place in 0.25% potassium permanganate for 30 min. or longer (up to 2 hr.) or in 1% solution for 20-30 min.
5. Wash in distilled water.
6. Bleach in 1% oxalic acid until colorless.
7. Wash in distilled water very thoroughly.
8. Stain in Mallory phosphotungstic acid hematoxylin (p. 1658) for 12-24 hr.
9. Wash out excess stain in 95% alcohol.
10. Dehydrate in 2 changes of absolute alcohol.
11. Blot and clear in 2 changes of pure xylol.
12. Mount in gum damar.

Cowdry mitochondria stain

1. Fix tissues in Regaud mixture (p. 1641). The commercial formalin may profitably be neutralized by saturation with magnesium carbonate. The mixture may be applied by immersion or injection, the latter being recommended for large objects. It should be changed every 4 days.
2. Mordant 8 days in 3% potassium dichromate, changing every second day.
3. Wash in running water overnight.
4. Dehydrate, clear, and embed in the usual manner.
5. Cut paraffin sections and pass slides to water in the usual manner.
6. Place in 1% potassium permanganate for 30 sec. or longer.
7. Place in 5% oxalic acid for 30 sec.
8. Rinse in several changes of distilled water for about 1 min. each. Incomplete washing prevents staining with fuchsin.
9. Stain in Altman aniline fuchsin (p. 1661). To stain, dry the slide with a towel, leaving the small area to which the section is attached. Cover the section with stain and heat over a small flame until strong aniline oil fumes come off; allow to cool; let the stain remain on the section about 6 min.; return the stain to the bottle.
10. Absorb most of the stain with a towel and rinse in distilled water so that the only remaining stain is in the section. If a large amount of stain is left, it will form a precipitate with methyl green. On the other hand, if too much stain is removed, the coloration of mitochondria will be faint.
11. Allow a few drops of 1% methyl green to flow over the section from a pipet, holding the slide over a piece of white paper so that colors may be seen. Apply the methyl green for about 5 sec. at first and modify as required. This is the crucial point of the method.
12. Drain off the excess stain, plunge into 95% alcohol for a few seconds, then rinse in absolute alcohol, clear in toluol, and mount in gum damar.

Note:

1. The methyl green may remove all the fuchsin, even when applied for only a short time. This is due to incomplete mordanting of the mitochondria by the chrome salts in fixation. It may be avoided by omitting the

permanganate and oxalic acid steps or by treating the section with 2% potassium dichromate for a few seconds just before staining. The purpose of potassium permanganate and oxalic acid is to remove the potassium dichromate.

2. The fuchsin may stain so intensely that the methyl green removes it imperfectly or not at all. This is due to too much mordanting. It may be corrected by prolonging the permanganate and oxalic acid treatment.

3. At times, after obtaining a good differentiation, the methyl green is washed out before the slide is placed in toluol, in which event omit the 95% alcohol and pass directly to absolute alcohol.

MYELIN SHEATH STAINS

Weigert-Pal method

The Weigert-Pal method is slow but very useful. Refer to p. 1708 for the method.

Spielmeyer method

The Spielmeyer procedure is a good stain for fine detail of myelinated fibers. Refer to the method on p. 1708 and Fig. 558, p. 1708.

Courville-Krajian method

Krajian myelin sheath stain has all the advantages of the longer methods, shows very fine detail of the fibrils, and can be completed in 1 hr. Refer to the method on p. 1709 and Fig. 559, p. 1709.

Pal-Kulschitzky method

Pal-Kulschitzky method usually yields satisfactory results and is valuable for rapid staining of myelin sheath. Refer to the method on p. 1709.

Morgan method

Morgan myelin sheath stain is a useful method to demonstrate myelin in paraffin sections. It is described on p. 1710.

NONMEDULLATED NERVE FIBERS

Ranson method

1. Fix segments of nerve for 48 hr. in a mixture of 99 ml. absolute alcohol and 1 ml. ammonium hydroxide.
2. Rinse tissues in distilled water.
3. Place in pyridine for 24 hr.
4. Place in many changes of distilled water for 24 hr.
5. Place in 2% silver nitrate for 3 days in the dark.
6. Rinse in distilled water.
7. Reduce for 24-48 hr. in 4% pyrogallol in 5% formalin.
8. Wash in water.
9. Dehydrate in graded alcohols, embed in paraffin, and cut sections.

This is a modification of the Cajal technic for neurofibrils. Nonmyelinated fibers appear black; myelinated axons, yellow; endoneurium, pale yellow.

NEUROGLIA FIBER STAINS

Cajal gold chloride and sublimate method

Fixative:

Formalin	15 ml.
Ammonium bromide	2 gm.
Distilled water	85 ml.

Gold chloride solution:

Distilled water	60 ml.
Bichloride of mercury	0.5 gm.
Gold chloride, 1% (Merck brown)	10 ml.

Fixing bath:

Sodium hyposulfite, saturated solution	5 ml.
Distilled water	70 ml.
Alcohol, 95%	30 ml.
Sodium bisulfite, saturated solution	5 ml.

Procedure:

1. Fix tissues for 2-10 days in the special fixative.
2. Cut frozen sections 20-25μ thick and place in distilled water to which a few drops of formalin have been added. After a quick wash transfer 4-6 sections into separate glass dishes of about 6 cm. diameter, each containing 15 ml. of the gold chloride solution.
3. After about 4 hr. the sections will have acquired an intense purple color. Place them in the fixing bath for 5-10 min.
4. Wash in 50% alcohol, transfer sections to slides, and blot in filter paper.
5. Dehydrate in 3 changes of absolute alcohol.
6. Clear in oil of origanum for 5 min.
7. Wash in 2 changes of pure xylol and mount in gum damar.

Precautions: Use pure brown gold chloride (Merck). If sections are left longer in the bath, good results may be obtained with ordinary gold chloride, but Cajal does not advise its use. Use only crystallized mercuric chloride. Temperature must not be lower than 12° C. for human brain tissue; it should be between 18° and 22° C. For cerebellum, medulla, and spinal cord, between 22° and 26° C. is preferred. Fixation for 3-15 days is preferable, but fibrous neuroglia may be fixed up to a year. The material must be in the fixative within 6 hr. after death.

Neuroglia are stained a dark purple on a much lighter purple background.

Globus modification of Cajal gold sublimate method for neuroglia

Globus provided a method of rendering tissue hardened in formalin available for staining with gold chloride which may be used if the tissue has not been in formalin too long.

1. Prepare frozen sections of formalin-fixed material 15-30μ thick.
2. Wash quickly in several changes of distilled water.
3. Place in 10% ammonium hydroxide for 24 hr. at room temperature or for a shorter period in an incubator.
4. Carry rapidly through 2 changes of distilled water.
5. Place in 10% solution of 41% hydrobromic acid for 2-4 hr.
6. Wash quickly in 2 changes of distilled water to which a few drops of ammonium hydroxide have been added. Place in Cajal gold chloride solution (p. 1739).
7. The sections assume an intense purple color after about 4 hr. Place 5-10 min. in Cajal fixing bath (p. 1739).
8. Wash in 50% alcohol, float, and transfer sections to slides. Blot in filter paper.
9. Dehydrate in 3 changes of absolute alcohol.
10. Clear in oil of origanum for 5 min.
11. Wash in 2 changes of pure xylol and mount in gum damar.

ASTROCYTES

Achucárro's fourth variant of Hortega

Tannin bromide solution:

Tannin	3 gm.
Ammonium bromide	1 gm.
Distilled water	100 ml.

Procedure:

1. Fix tissues in 10% formalin.
2. Cut frozen sections and wash thoroughly in distilled water.
3. Place for 10 min. in tannin-bromide solution at 45°-50° C.
4. Before the sections cool wash in 20 ml. distilled water containing 2 drops of ammonium hydroxide, until they have regained their flexibility and transparency.
5. Place at once in dilute Bielschowsky silver (1:10) (p. 1736) until sections assume a yellowish color.
6. Transfer to a fresh dish as soon as the liquid becomes turbid (use about 3 dishes).
7. Wash in distilled water.
8. Reduce in 20% formalin (neutralized with chalk).
9. Wash and dehydrate in absolute alcohol.
10. Clear in carbolxylol, wash in xylol, and mount in gum damar.

GLIA FIBRIL STAINS

Mallory phosphotungstic acid hematoxylin

Mallory phosphotungstic acid hematoxylin is particularly useful for demonstrating fibrin and neuroglia, fibroglia, and myoglia fibrils. It also brings out the detailed structure in mitotic figures, including the spindles and centrosomes. See p. 1734.

Alzheimer method for glia fibril

1. Fix tissues for at least 1 wk. in 10% formalin. Cut frozen, paraffin, or celloidin sections 5-10μ thick. Mount on slide and carry through customary hydrating solutions to distilled watter.
2. Mordant in a mixture of 19 ml. 1% chromic acid and 1 ml. glacial acetic acid.
3. Wash quickly but thoroughly in distilled water.
4. Place sections for 1 hr. in a paraffin oven at 55° C. in a saturated aqueous solution of acid fuchsin in a jar.
5. Wash in water until no more color comes off.

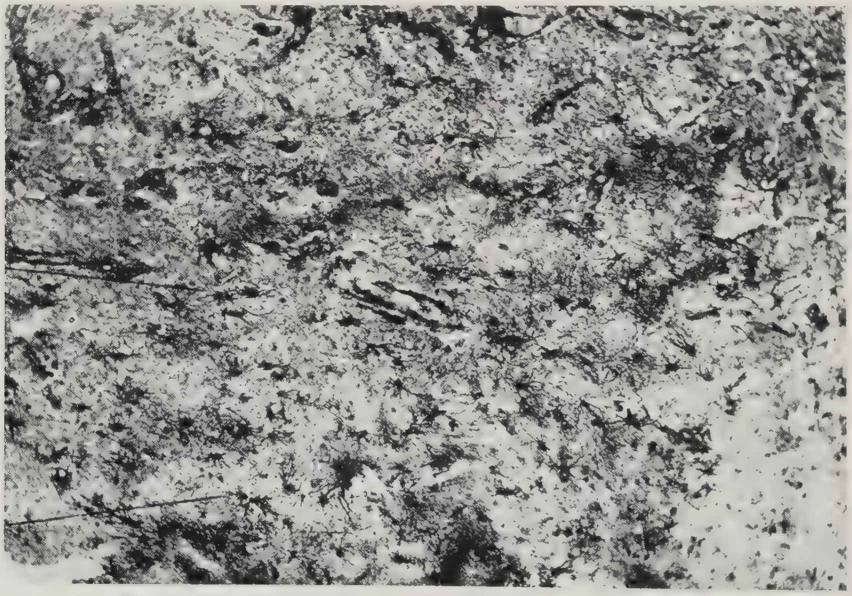


Fig. 579. Astrocytes in glioblastoma, Achucárro method. Frozen section. Lines lead to astrocytes. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

6. Agitate sections in a mixture of 30 ml. saturated alcoholic picric acid and 60 ml. distilled water.
7. Wash in 2 changes of distilled water.
8. Place sections in saturated aqueous solution of light green, which has been diluted with an equal amount of distilled water, for about 30 min.
9. Wash in water.
10. Dehydrate in 95% alcohol and then in 2 changes of absolute alcohol.
11. Clear in 2 changes of pure xylol and mount in gum damar.

Glia fibrils stain bright red against a very pale green background.

Heidenhain method for glia fibril

1. Fix tissues in 10% formalin.
2. Embed in paraffin in the usual manner.
3. Cut sections 5-10μ thick.
4. Remove the paraffin by xylol and place in 2.5% (violet) iron alum solution for 6-12 hr.
5. Wash in distilled water rapidly but thoroughly.
6. Stain in 0.5% old aqueous hematoxylin (p. 1658) 30 min. or longer.
7. Wash in distilled water.
8. Differentiate in a weak solution of iron alum, controlling the differentiation under a microscope.
9. Wash thoroughly in tap water.
10. Rinse in distilled water.
11. Dehydrate in 95% alcohol; follow with absolute alcohol.
12. Blot and clear in 2 changes of pure xylol.
13. Mount in gum damar.

OLIGODENDROGLIA STAINS

Hortega silver carbonate method

Hardening solution:

Hydrobromic acid, 40%	5 ml.
Distilled water	95 ml.

Strong silver carbonate solution:

Silver nitrate, 10%	5 ml.
Lithium carbonate, saturated solution	20 ml.
Ammonium hydroxide	Sufficient to dissolve precipitate
Distilled water	75 ml.

1. When the 2 chemicals are combined, there is a voluminous precipitate of silver carbonate. The ammonia should then be added drop by drop, stirring all the while, until the precipitate disappears and ammoniacal silver goes into solution. A small amount of black, dustlike precipitate remains undissolved and may be filtered off.
2. The solution keeps well in the dark.

Procedure:

1. Harden tissues for 12-48 hr. in Cajal fixative, p. 1739.
2. Bromurate by heating blocks in fresh hardening solution for 10 min. at 45°-50° C.
3. Cut frozen sections 15-20μ thick.
4. Wash in 2 changes of distilled water. Add 10 drops of ammonium hydroxide to the first water.
5. Impregnate. Place sections in strong silver carbonate solution and leave there for 1-5 min.
6. Wash. Agitate gently for about 15 sec.
7. Reduce by dipping sections in 1% formalin but do not agitate.
8. Wash and tone in 0.2% aqueous gold chloride.
9. Fix in 5% sodium hyposulfite for 1 min.
10. Wash in distilled water, dehydrate, clear, and mount in the usual manner.

Oligodendroglia will stain selectively. There may be faint staining of astrocytes, and microglia may stain well.

Penfield modification of Hortega silver carbonate method

1. Harden tissues in ammonium bromide solution (p. 1739) for 2-48 hr. Blocks should be not more than 3 mm. thick.
2. Place blocks in 95% alcohol for 36-48 hr.
3. To eliminate the alcohol wash blocks about 4 hr. in several changes of distilled water in large volume.
4. Cut frozen sections. If the alcohol is not sufficiently washed out, cutting will be difficult.
5. Pass through 2 changes of distilled water.
6. Leave in strong silver carbonate (p. 1741) for 15 min.-2 hr., as determined by trial. The most favorable time to remove sections is when they are just beginning to turn brown.
7. Plunge directly into 1% formalin and agitate at once.
8. Wash in water.
9. Tone in 0.2% gold chloride solution until gray.
10. Fix in 5% sodium hyposulfite for 1 min.
11. Wash, dehydrate, clear, and mount as usual.

This method, like that of Hortega, is variable, although the results are brilliant when successful.

MICROGLIA STAIN

Hortega silver carbonate method for microglia

1. Fix thin pieces of tissue, 2 or 3 mm. thick, in Cajal fixative (p. 1739) 1 or 2 days at 35° C. or 2-4 days at room temperature.
2. Warm the pieces of tissue for 10 min. at 50°-55° C. in a fresh volume of the fixative.
3. Make frozen sections 25-30μ thick.
4. Rinse quickly in 2 changes of distilled water containing 2 drops of ammonium hydroxide for each 50 ml. water.
5. Treat for 10 min. at 15°-20° C. in ammoniacal silver carbonate solution. To make this solution add 5 ml. 10% silver nitrate to 20 ml. 5% sodium carbonate, add ammonium hydroxide drop by drop until the yellow precipitate is just dissolved, and then add 15 ml. water.
6. Reduce in 10% acid-free formalin. Move sections during this process. A simple expedient is to place them in a large Petri dish and blow on the fluid gently.

7. Wash in distilled water.
8. Tone in 0.2% yellow gold chloride solution until the sections turn gray, usually 10-15 min.
9. Treat in 5% sodium hyposulfite for 1 min.
10. Dehydrate in 95% alcohol.
11. Clear in **carbolxylol-creosote** mixture:

Phenol	10 gm.
Xylol	80 ml.
Creosote, pure	10 ml.

12. Mount in gum damar.

Microglia stain deeply; fibrous neuroglia and glia fibers stain faintly.

OLIGODENDROGLIA AND MICROGLIA STAIN

Penfield combined method

Hortega weak ammoniacal silver carbonate solution:

Silver nitrate, 10%	5 ml.
Sodium carbonate, 5%	20 ml.
Ammonium hydroxide	Sufficient to dissolve precipitate
Distilled water	to make 75 ml.

1. Add the ammonium hydroxide drop by drop until the precipitate is just dissolved, stirring constantly. Do not add too much ammonia. A fine black sediment should remain, which does not resemble the more voluminous precipitate of silver carbonate.
2. Filter off this fine sediment.
3. The solution may then be preserved in a dark bottle for long periods.

Procedure:

1. Harden tissue in 10% formalin or formol-ammonium bromide (p. 1739) for an indefinite period. About a week's fixation in formalin gives excellent results.
2. Cut frozen sections 20μ thick and place in 1% formalin or distilled water. Through the succeeding steps handle sections with a glass rod.
3. Place in a dish of distilled water to which 10-15 drops of ammonium hydroxide have been added and cover to prevent escape of the ammonia. Leave in this solution overnight to remove the formalin.
4. Transfer directly to 5% aqueous hydrobromic acid (41%). Place in an incubator at 38° C. for 1 hr.
5. Wash in 3 changes of water.
6. Place in 5% sodium carbonate for 1 hr. Sections may remain in this solution 3-6 hr. without ill effect.
7. Pass, with or without washing, directly into Hortega weak silver carbonate and leave for 3-5 min. At times the sections may be left until they begin to turn yellowish gray. Then transfer to 1% formalin (reducer). Control the duration in the silver solution by taking out a section at intervals of 1-2 min. and examining under a microscope. The sections should turn a smooth gray color in the reducer.
8. Plunge into 1% formalin and agitate.
9. Wash in distilled water.
10. Tone in 1:500 gold chloride at room temperature until all yellow tint disappears and the sections are a smooth bluish gray.
11. Treat in 5% sodium hyposulfite.
12. Wash in distilled water.
13. Float onto slides and flatten out with a teasing needle.

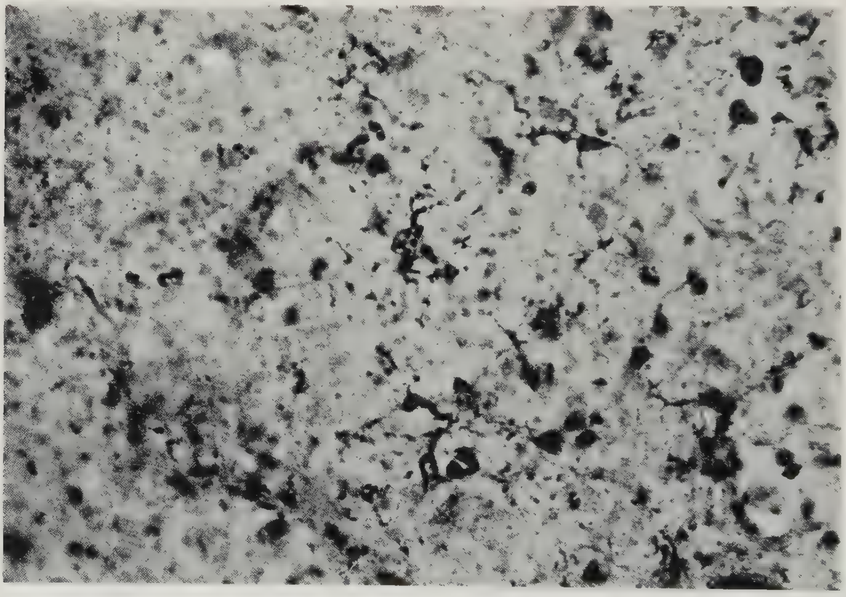


Fig. 580. Early changes of microglia in old gunshot wound of brain. Penfield combined method. Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

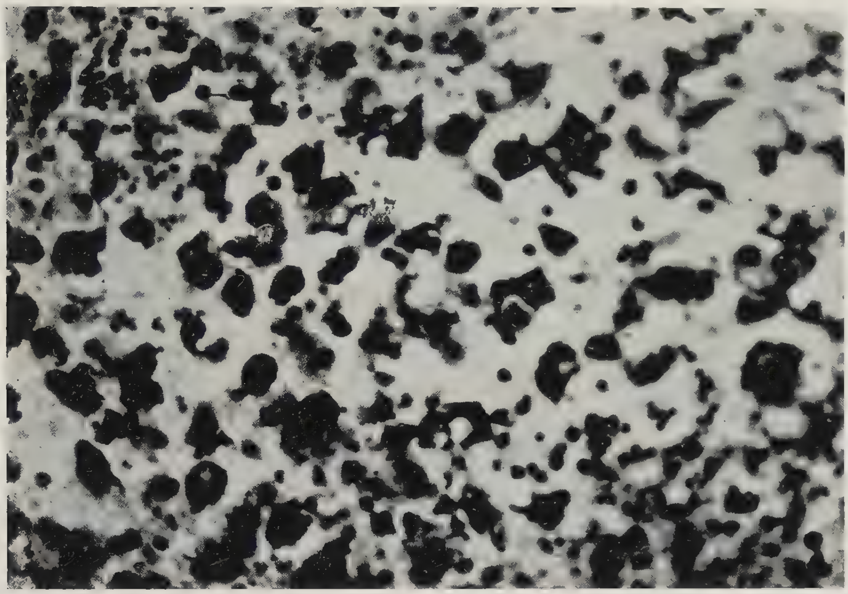


Fig. 581. Microglia developing phagocytes at margin of a brain wound. Penfield combined method. Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

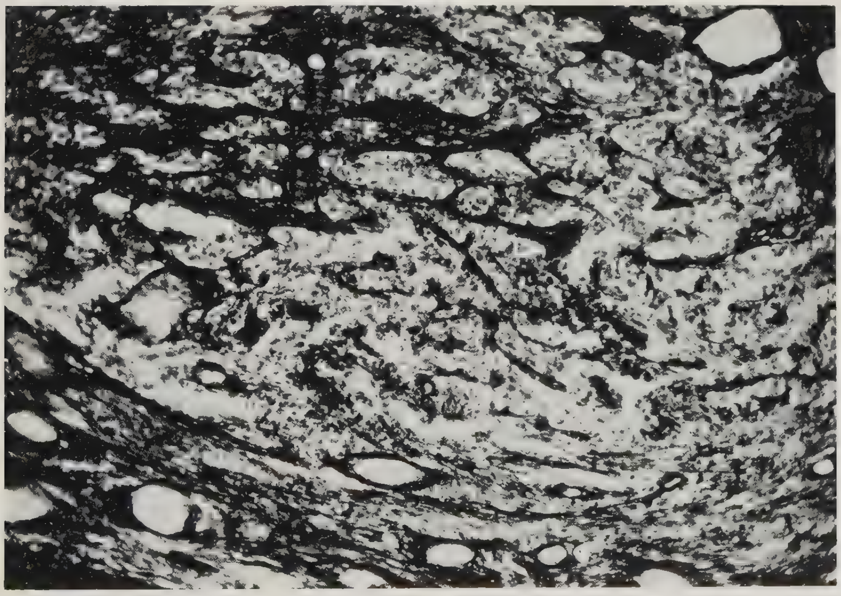


Fig. 582. Reticulum in a ganglion cell tumor. Krajian reticulum stain. Reticular fibers stain black. Frozen section. (From Krajian, A. A., and Gradwohl, R. B. H.: Histopathological technic, St. Louis, 1952, The C. V. Mosby Co.)

14. Dehydrate in 2-4 changes of 95% alcohol from a drop bottle.
15. Follow with a few drops of carbolxylol. When clear, drain the slides and blot immediately with 2 thicknesses of fine filter paper.
16. Mount in gum damar.

The yellow variety of gold chloride is less expensive than the brown and seems to be preferable for toning.

This method is highly recommended.

RETICULUM STAINS

Use Perdrau modification of the Bielschowsky stain, the Krajian rapid method for staining reticulum and collagen fibers in frozen sections, or the Foot method for staining reticulum in paraffin sections.

CONNECTIVE TISSUE STAINS

Use Mallory aniline blue stain or the Van Gieson method. The Heidenhain azocarmine stain is excellent for demonstrating minute details of connective tissue.

Chapter 93

Otohistologic technics

ARAM A. KRAJIAN

USE OF RADIOACTIVE METALLIC SALTS AND OTHER RARE METALLIC SALTS AND CHEMICALS

In otohistology, most present-day staining is done with hematoxylin and aniline dyes; some selective stains are used, such as Mallory aniline blue and van Gieson stains to demonstrate connective tissue.

Because of prolonged immersion of temporal bones in strong acids and subsequent dehydrating and embedding technics, the resulting microscopic sections often fail to show the desired nuclear stain or do not differentiate the various elements of the tissue, such as smooth and striated muscle fibers, connective tissue fibers, ganglion cells, bone tissue, fat globules, fibrin, hair cells, lacuna, caniculi, osteocytes with their processes, etc.

In the histology laboratories of a number of hospitals and universities some metallic salt, such as mercuric chloride, is used as a mordant in Zenker and Helly solutions. Silver nitrate is extensively used in neuropathologic work for the demonstration of nerve fibers, basal ganglia, ganglion cells, nonmedullated nerve fibers, glia, astrocytes, microglia, oligodendroglia, peripheral nerve fibers, and reticulum. Gold chloride is used to demonstrate nerve endings. In our efforts to develop selective staining technics for temporal bone sections, we have made use of hitherto untried radioactive metallic salts such as thorium and uranium nitrate, as well as other metallic salts such as strontium nitrate, bismuth nitrate, iodine, gold chloride, stannous chloride, lead nitrate, magnesium nitrate, cobalt nitrate, molybdenum nitrate, nickel nitrate, palladium nitrate, zirconium nitrate, zinc nitrate.

Several mordants have been developed with the combination of the above metallic salts and other chemical elements. Among the mordants produced, the most effective was thorium-ferric ammonium sulfate combination. Utilizing this mordant, the following new selective stains were developed.

Method 1—New selective hematoxylin

A new selective hematoxylin is described for demonstrating smooth and striated muscle fibers, elastic and connective tissue, bone tissue, nerve fibers, plasma cells, erythrocytes, and leukocytes.

Prepare celloidin sections as on p. 1753.

Reagents:

1. Strong iodine solution.
    (a) Thoroughly mix 2 gm. iodine and 4 gm. potassium iodide in a mortar. Dissolve in 5 ml. distilled water; then add 95 ml of distilled water.
2. Thorium-ferric solution.

Thorium nitrate	4 gm.
Ferric ammonium sulfate	10 gm.
Distilled water	100 ml.

3. Creosote-xylol.
    (a) Equal parts of beechwood creosote and xylol.

Procedure:

1. Place sections in 10% strontium nitrate for 5 min.; then rinse in tap water.
2. Treat in strong iodine solution for 5 min.
3. Wash in 3 changes of tap water and mordant in thorium-ferric solution for 10 min.
4. Wash in 3 changes of distilled water and stain in Harris hematoxylin (p. 1658) for 30 min.
5. Wash and treat for 3 min. in 3% sodium cyanide.
6. Wash in tap water and differentiate in 5% solution of thorium nitrate for 2-5 min. This

Text continued on p. 1750.

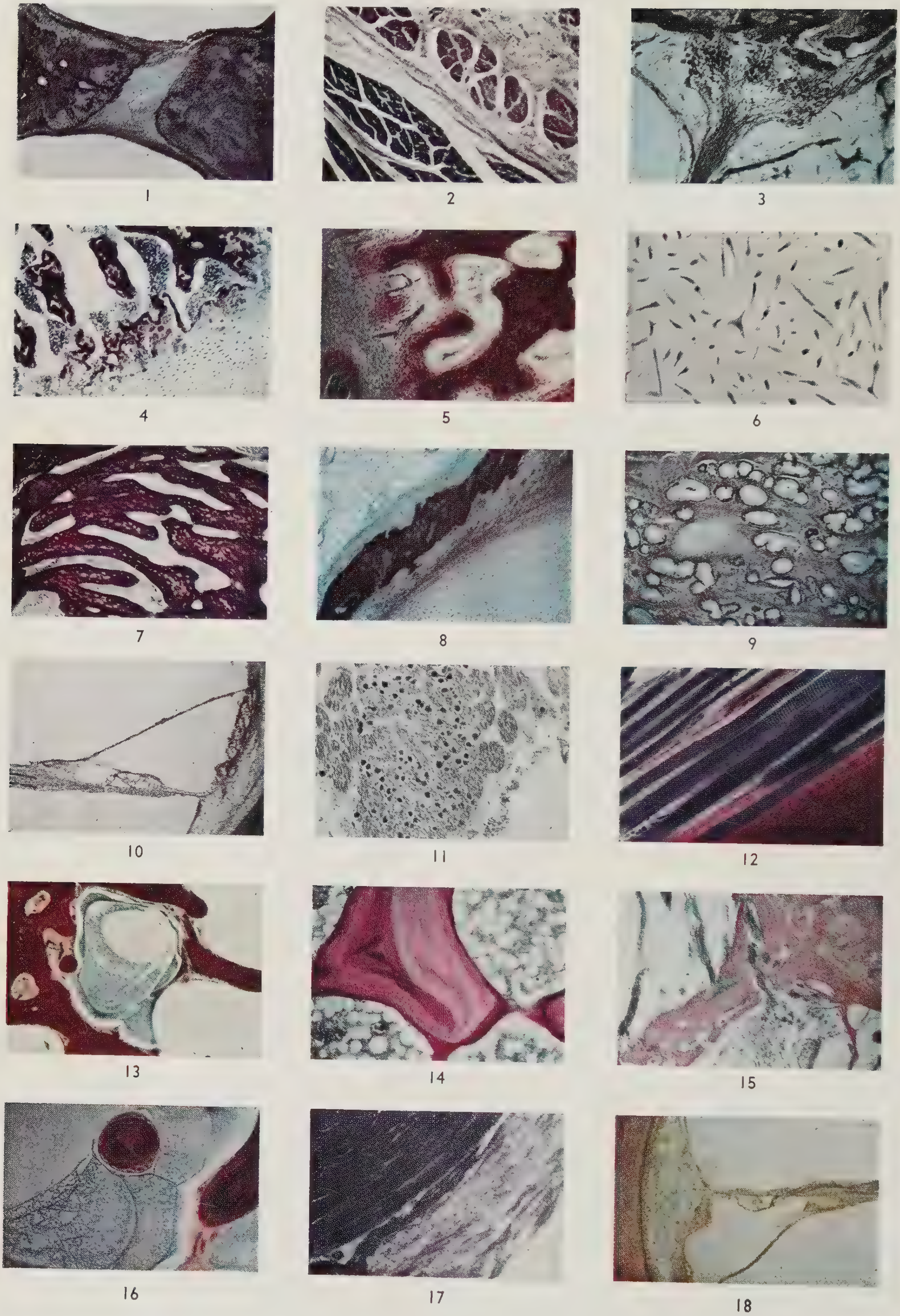

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Fig. 583. Celloidin sections of temporal bone and frozen sections of brain.

Fig. 583

Celloidin sections of temporal bone
Method 1: Temporal bone section (selective hematoxylin stain)
1. Bone tissue, magenta; cartilage, light red; tendon, grayish red.
2. Neck muscle. Smooth muscle fibers, dark red; striated muscle fibers, dark blue; connective tissue, light red.
3. Bone tissue, magenta; cochlear nerve, blue.
4. Bone tissue, magenta; marrow, dark grayish blue; cartilage, light red.
5. Bone tissue, magenta; connective tissue cells, blue.
6. Cartilage, light pink; cartilage cells, dark blue.
7. Bone tissue, magenta; marrow, blue gray.
8. Bone tissue, magenta; cartilage, pinkish blue; connective tissue, red.
9. Mucous glands. Secretion, gray blue; connective tissue, red; cartilage, reddish blue.
10. Spiral ligament, purplish blue; hair cells, purple; connective tissue, red; tectorial membrane, light pink.
11. Cross section of acoustic nerve, purplish blue.

Method 2: Temporal bone section (new differential nerve stain)

12. Striated muscle fibers, purple; connective tissue, red.
13. Bone tissue, magenta red; fibrin, grayish blue.
14. Bone tissue, magenta red; marrow, grayish blue.
15. Bone tissue, rose red; cochlear nerve fibers, purplish blue.
16. Bone tissue, magenta red; fibrin, grayish blue.
17. Acoustic nerve, purplish blue.
18. Cochlear cavity. Bone tissue, rose red; spiral ligament, brownish red; connective tissue, pink; tectorial membrane, creamy yellow.

Fig. 583 (cont'd)

Celloidin sections of temporal bone (cont'd)
Method 3: Temporal bone section, chemical coloration (gold chloride)
19. Bone tissue, dark blue; marrow, rose.
20. Cochlear bone, dark blue; spiral ligament, light rose; cartilage cells, light rose.
21. Bone tissue, dark blue; cartilage, light blue; cartilage cells, rose.
22. Bone tissue, dark blue; nerve fibers, rose.

Method 4: Temporal bone section, chemical coloration (iodine combination method)
23. Neck muscle. Smooth muscle fibers, dark blue; connective tissue, light blue; striated muscle fibers, dark brown.
24. Striated muscle fibers, dark brown; connective tissue, light blue.
25. Bone tissue, dark blue; nerve fibers, brown; cartilage, light greenish blue; striated muscle fibers, dark brown.
26. Arterial wall, brown; elastic fibers, dark brown; connective tissue, dark blue.
27. Bone tissue, deep greenish blue; marrow, light brown.
28. Striated muscle fibers, dark brown; connective tissue, blue; cartilage, light blue; mucous glands, brown; connective tissue, light blue.
29. Neck muscle. Smooth muscle fibers, dark blue; striated muscle fibers, magenta red (azocarmine counterstain).
30. Striated muscle fibers, magenta red (azocarmine counterstain).

Method 5: Temporal bone section, chemical coloration (zinc permanganate)
31. Compact bone, brown; haversian canal nerve fibers, brown; connective tissue, greenish blue; lacuna, bright blue; nucleus of osteocyte, dark brown; osteocytic processes, brown; devitalized osteocytes, dark brown.
32. Temporal bone. Vestibular membrane, light greenish blue; vestibular nerve, brown; tectorial membrane, light greenish blue; hair cells, dark brown.
33. Mucous glands. Glands, brown; bone tissue, dark blue; connective tissue, light blue; cartilage, light grayish blue; striated muscle fibers, dark brown.

Frozen sections of brain
Method 6: New myelin sheath stain for frozen sections
34. Brain tissue. Myelinated fibers, blue black.
35. Brain section. Myelinated fibers, blue black.
36. Brain section. Brain cells, light pink with brown nuclei.

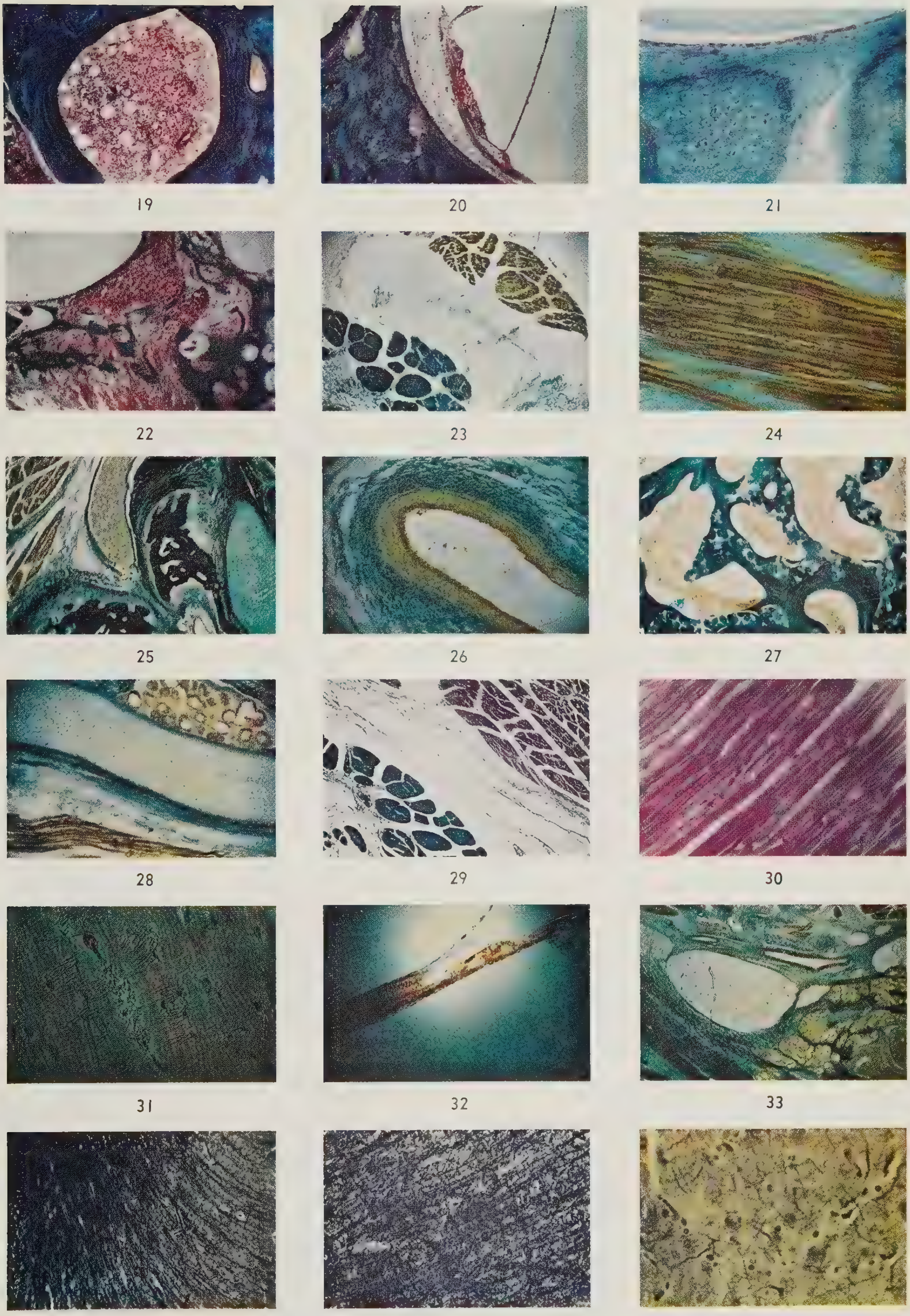

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Fig. 583 (cont'd)

treatment depends on the strength of the solution and the thickness of the sections. It should be controlled under a microscope or until the celloidin around the section clears.

7. Wash in tap water and transfer the section to a glass slide.
8. Blot dry and dehydrate with benzol for 1 min.
9. Pour off the benzol, blot, and dip once in thin celloidin (p. 1666).
10. Clear for 3 min. in creosote-xylol and then 5 min. in pure xylol.
11. Mount in gum damar or Permount.

All solutions are stable and can be used repeatedly.

Bone tissue stains shades of magenta; bone marrow cells, light gray; bone cells, dark blue; red blood cells, grayish blue; connective tissue, shades of light red; striated muscle fibers, blue black; smooth muscle fibers, red; fatty acid crystals, gray blue; walls of blood vessels, dark blue; nerve fibers, deep blue; plasma cell nuclei, blue with cytoplasmic granules; calcium, gray blue; elastic fibers, dark blue; cartilage, light pink.

Method 2—
New differential nerve stain

The following stain demonstrates fibrin, smooth and striated muscle fibers, elastic and connective tissue, bone tissue, hair cells, erythrocytes, and leukocytes by the use of **thorium-ferric ammonium sulfate mordant** and with the addition of lithium and bismuth nitrate.

Prepare colloidin sections (p. 1753).

Reagents:

1. Phospho-hematoxylin.
   Hematoxylin crystals . . . . 0.1 gm.
   Distilled water . . . . 5 gm.
   (a) Dissolve with the aid of heat.
   (b) Mix in 100 ml. 2% phosphotungstic acid.
   (c) For immediate use, ripen by adding 10 ml. 0.25% potassium permanganate.

Procedure:

1. Place temporal bone section in warm (30° C.) 10% lithium nitrate for 5 min.
2. Wash in 2 changes of tap water.
3. Mordant in thorium ferric solution at 30° C. for 10 min. (p. 1745).
4. Wash in 3 changes of tap water and place in warm (30° C.) 2% bismuth nitrate for 10 min. The bismuth solution should be filtered.
5. Wash and stain in warm (30° C.) phospho-hematoxylin.
6. Wash twice in tap water and treat in 2% magnesium nitrate solution for 1 min.
7. Wash and transfer section onto glass slide.
8. Blot dry in filter paper and dehydrate in benzol for 1 min.
9. Pour off benzol, blot dry, and dip once in thin celloidin (p. 1666).
10. Partially clear in creosote-xylol, equal parts, for 5 min.
11. Clear in pure xylol for 5 min. and mount in gum damar.

All the solutions are stable and can be used repeatedly.

Fibrin stains gray blue; nerve fibers, dark blue; ganglion cells, dark rose; elastic fibers, violet blue; striated muscle fibers, violet blue; smooth muscle fibers, dark red; connective tissue, cream color; bone tissue, magenta and red; hair cells, violet blue; walls of fat globules, pale straw; leukocytes, purple blue; erythrocytes, light gray.

The purpose of staining is to recognize the various elements of tissue and the cell components. Some stains are strictly solution phenomena, such as staining neutral fats with oil-soluble dyes (scharlach R or oil red); others are strictly chemical phenomena, such as the production of Prussian blue reaction by hemosiderin in the presence of acid and potassium ferrocyanide; others depend on the presence of mordants, such as hematoxylin with ferric and aluminum salts or numerous other metallic salts.

While investigating the feasibility of procuring a chemical coloration without the aid of dyes on temporal bone sections, we have succeeded in developing 3 new formulas by making use of the following metallic salts and chemicals.

1. Lithium nitrate, iodine solution, gold chloride, orcinol, phloroglucin, thorium-ferric ammonium sulfate, potassium, ferrocyanide, and thorium nitrate solution.
2. Lead nitrate, iodine solution, ferric ammonium sulfate, and potassium ferrocyanide.
3. Zirconium nitrate, zinc nitrate, ferric ammonium sulfate, potassium ferrocyanide, and potassium permanganate.

Method 3—
Chemical coloration of temporal bone sections

Bone tissue, smooth and striated muscle fibers, connective tissue, and nerve fibers are identified by the following **gold chloride method.**

Prepare colloidin sections (p. 1753).

Reagent:

1. Thorium-ferric solution.
   (a) Dissolve 5 gm. thorium nitrate in 100 ml. 10% ferric ammonium sulfate.

Procedure:

1. Place section in 10% lithium nitrate for 3 min.
2. Wash in 2 changes of tap water and treat in strong iodine solution (p. 1745).
3. Wash in 3 changes of tap water and treat in 10% lithium nitrate for 10 sec., dipping section in and out, and wash 3 times.

4. Treat in 1% gold chloride for 3 min. and wash 3 times.
5. Treat in 10% lithium nitrate for 10 sec., dipping section in and out, and wash 3 times.
6. Place in 2% orcinol solution for 3 min. and wash 3 times.
7. Treat in 10% lithium nitrate for 10 sec. and wash 3 times.
8. Place in 5% phloroglucin for 3 min. and wash 3 times.
9. Treat in 10% lithium nitrate for 10 sec., dipping the section in and out, and wash 3 times.
10. Mordant in thorium-ferric solution for 2 min. and wash 3 times.
11. Treat in 10% lithium nitrate for 10 sec., dipping the section in and out, and wash 3 times.
12. Treat in 10% potassium ferrocyanide solution for 10 sec. and wash 3 times.
13. Treat in 10% lithium nitrate for 10 sec., dipping the section in and out, and wash 3 times.
14. Treat in 1% thorium nitrate for 45 sec. or until the background is an even blue and wash 3 times.
15. Treat for a few seconds in 10% lithium nitrate and wash.
16. Transfer the section onto a glass slide and blot dry in filter paper.
17. Dehydrate, pouring benzol over the section for 1 min.
18. Pour off the benzol, blot dry, and dip once in thin celloidin (p. 1666).
19. Partially clear in creosote-xylol (equal parts) for 5 min.
20. Clear in pure xylol for 5 min. and mount in gum damar or Permount.

All the solutions are stable and can be used repeatedly. Gold chloride and orcinol should be filtered before use.

Nerve stains rose color; ganglion cells, dirty rose; bone tissue, shades of blue; striated muscle fibers, dark rose; smooth muscle fibers, dirty blue; red blood cells, dark red; connective tissue, greenish blue; leukocytes, purplish blue; arterial wall, dark red; capsule of fat globules, cream.

This method, besides its colorful reaction to different elements, is also an excellent connective tissue stain, similar to Mallory aniline blue. It also differentiates smooth and striated muscles with different colors.

Method 4—
New chemical coloration method—iodine combination technic

This method demonstrates nerve fibers, smooth and striated muscle fibers, connective tissue, bone tissue, blood vessels, bone marrow, and other elements.

Prepare celloidin sections (p. 1753).

Reagent:

1. Terpineol-xylol.
   (a) Use equal parts and mix.

Procedure:

1. Place sections in 10% lead nitrate for 5 min.
2. Wash in 3 changes of distilled water and mordant in 10% strontium nitrate for 5 min.
3. Wash and treat in 5% phloroglucin for 5 min.
4. Wash and place in strong iodine (p. 1745) for 5 min.
5. Wash 3 times and place in 15% ferric ammonium sulfate for 5 min.
6. Wash twice and place in strong iodine for 5 min.
7. Wash 3 times and place in 15% ferric ammonium sulfate for 5 min.
8. Wash twice and place in strong iodine for 5 min.
9. Wash 3 times in tap water, rinse twice in distilled water, and place in 10% potassium ferrocyanide for 15 sec.
10. Rinse in 2 changes of distilled water, then in tap water, and dip for 5 sec. in 15% ferric ammonium sulfate.
11. Wash and place in strong iodine for 3 min.
12. Wash 3 times in tap water and transfer section onto a glass slide.
13. Blot dry in filter paper; then pour benzol over the section for 1 min.
14. Dip once in thin celloidin (p. 1666).
15. Clear in terpineol-xylol for 5 min.
16. Mount in gum damar or Permount.

All the solutions are stable and can be used repeatedly.

Bone tissue stains blue; connective tissue, greenish blue; smooth muscle fibers, deep blue; striated muscle fibers, golden yellow; walls of blood vessels, yellow; red blood cells, straw; nerve fibers, yellow.

Method 5—
New chemical coloration method—zinc-permanganate technic

This method demonstrates haversian canals, osteocytes with their processes, nerve and connective tissue fibers, smooth and striated muscle fibers, epithelium, spiral ganglion, vascular wall, etc.

Prepare celloidin sections (p. 1753).

Reagent:

1. Zinc-permanganate.

Zinc nitrate	2 gm.
Potassium permanganate . .	0.25 gm.

   (a) Dissolve in a mixture of 3 drops of hydrochloric acid, concentrated, in 40 ml. distilled water.

Procedure:

1. Place section in 5% zirconium nitrate for 3 min. and wash 3 times.
2. Treat for 3 min. in zinc-permanganate and wash 3 times.

3. Treat in 5% zinc nitrate, agitating the section 15-20 times, and wash 3 times.
4. Place in 5% zirconium nitrate for 2 min. and wash 3 times.
5. Treat in zinc-permanganate solution for 2 min. and wash 3 times.
6. Treat in 5% zinc nitrate, agitating 15-20 times, and wash 3 times.
7. Place in 5% zirconium nitrate for 1 min. and wash 3 times.
8. Treat in zinc-permanganate for 1 min. and wash 3 times.
9. Agitate 15-20 times in 5% zinc nitrate and wash 3 times.
10. Place in 10% dioxane for 1 min. (5 ml. dioxane in 45 ml. distilled water) and wash 3 times.
11. Differentiate in 2% phosphotungstic acid for 1 min. and wash 3 times.
12. Treat in 10% potassium iodide for 1 min. and wash 3 times.
13. Mordant in 10% ferric ammonium sulfate for 4 min. and wash 3 times.
14. Treat in 10% potassium ferrocyanide for 20 sec. and wash 3 times.
15. Place in 2% phloroglucin for 1 min. and wash 3 times.
16. Mordant in 10% ferric ammonium sulfate once more for 2 min. and wash 3 times.
17. Dip section 5 times in 10% potassium ferrocyanide and wash 3 times.
18. Treat in 2% phloroglucin for 1 min. and wash 3 times.
19. Treat in 5% zinc nitrate for 1 min. and wash 3 times.
20. Transfer section onto a glass slide and blot in fine filter paper until completely dry.
21. Dehydrate section with benzol, pouring the benzol over the section.
22. Pour off the benzol and blot several times.
23. Dip in thin celloidin (p. 1666) 3 times and blow the breath over the section until dry.
24. Flatten the section by blotting in filter paper 3 or 4 times and dehydrate with anhydrous isopropanol for 1 min.
25. Pour off isopropanol and clear the section in neutral xylol for 5 min.
26. Mount in gum damar.

Lacuna stains brilliant blue; compact bone, greenish yellow; nucleus of osteocytes, brownish; osteocyte processes, dark brown; nerve fibers, brown; connective tissue, blue; striated muscle fibers, dark brown; smooth muscle fibers, greenish blue; vascular walls, brown; epithelium, yellow; erythrocytes, golden yellow; spiral ganglion, dark brown; spiral ligament, greenish yellow.

Method 6—New myelin sheath stain for frozen sections of human and animal central nervous system tissue

The value of frozen sections for demonstrating tissue pathology has recently been emphasized, and the frozen section technic is becoming more generally recognized as its use in large hospitals and clinics has increased. Speed, economy, preservation of cellular structure, and absence of shrinkage are some of the advantages of the frozen section technic.

Since many experiments in otology are conducted on animals, it is important to recognize the histopathologic changes produced by these experiments on the central nervous system by the use of a suitable selective stain.

Present-day methods of staining are performed on celloidin sections. Embedding and sectioning celloidin sections is time-consuming and expensive. We have adopted the frozen section technic and have developed a new rapid and satisfactory method of staining myelin sheath in tissues of the central nervous system.

Reagents:

1. Hematoxylin.
   (a) Dissolve 10 gm. hematoxylin crystals in 100 ml. 95% ethyl alcohol.
   (b) Incubate 10 days or longer.
2. Thorium-ferric solution, 2%.
   (a) Add 1 part of strong thorium-ferric ammonium sulfate (p. 1750) to 5 parts water.
3. Aniline-xylol.
   (a) Equal parts of aniline oil and xylol.

Procedure:

1. Cut frozen sections 10-15μ thick and place in Cellosolve (ethylene glycol monoethyl ether) for 15 min.
2. Wash 3 times in tap water and carry to strong iodine solution (p. 1745) for 5 min. and wash 3 times.
3. Place in 10% potassium iodide solution for 5 min. and wash 3 times.
4. Mordant in a mixture of equal volumes of 2% antimony trichloride and 10% aluminum nitrate solution for 10 min. at 40° C. and wash 3 times.
5. Mordant in thorium-ferric solution (p. 1745) for 15 min. at 40° C.
6. Wash 3 times and stain in ripe hematoxylin, mixing 3 ml. ripe hematoxylin in 15 ml. tap water, for 30 min. at 40° C.
7. Partially decolorize in 3% sodium cyanide solution for 2 min. and wash 3 times.
8. Carry the decolorization further, treating in 2% thorium ferric solution for 1 min. and wash 3 times.
9. Differentiate further in 3% sodium cyanide for 2-4 min. or until the gray matter appears grayish. This step should be controlled under a microscope. If the gray matter is not completely clear, carry section once more to dilute thorium-ferric for 15 sec. and then for 2 min. in 3% sodium cyanide.
10. Wash 3 times and transfer the section onto a glass slide.
11. Blot dry and dehydrate with benzol, pouring the benzol over the section for 1 min.
12. Pour off the benzol, blot, and dip the section once into thin celloidin (about 7 ml. thick celloidin dissolved in 100 ml. ether-alcohol, equal volume).

13. Clear in aniline-xylol, pouring the mixture over the section, for 5 min.
14. Clear in pure xylol for 5 min. and mount in gum damar or Permount.

All the solutions are stable and can be used repeatedly.

Cellosolve may be used twice. Ripe hematoxylin solution should be prepared fresh each time.

Myelinated fibers stain dark blue; nerve cells, cytoplasm light gray, nuclei blue; red blood cells, gray blue; gray matter, light grayish pink.

NEW DECALCIFICATION METHOD FOR TEMPORAL BONES

The following method employs a controlled shaking machine and modified decalcifying fluid.

Reagent:

1. Decalcifying solution. Refer to p. 1644.

Technic of decalcification:

1. Place temporal bones in a 200 ml. specimen bottle and fill with decalcifying solution, pouring the solution on slowly. As the fluid contacts the bone, immense numbers of bubbles arise. Wait until the bubbles disappear; then stopper the bottle tightly and place on a shaking machine at 35 oscillations/min. The shaking machine is the standard Kahn shaker controlled with Variac.*
2. Change the solution twice at 8 hr. intervals, and prick the specimen superficially with a needle once a day. It requires 8-12 changes for a temporal bone to be completely decalcified, depending on the amount of calcium present or the hardness of tissue due to other causes.

The method is also applicable to smaller bones removed at surgery or autopsy, when it requires only 1 or 2 changes for complete decalcification.

3. After complete decalcification, the specimen should be neutralized by placing in 1% lithium carbonate for 24 hr. Then wash for 16-24 hr. under gently running water and place in 40% ethyl alcohol until ready for dehydration and embedding.

Dehydrating:

1. Place specimen in 50% alcohol on the shaker for 2½ hr.
2. Place specimen in 60% alcohol on the shaker for 2½ hr.
3. Place specimen in 70% alcohol on the shaker for 2½ hr.
4. Place specimen in 80% alcohol on the shaker for 2½ hr.
5. Place specimen in 95% alcohol on the shaker for 2½ hr.
6. Place specimen in 95% alcohol on the shaker for 2½ hr.
7. Place specimen in absolute alcohol on the shaker for 2½ hr.
8. Place specimen in absolute alcohol on the shaker for 2½ hr.
9. Place specimen in absolute alcohol and ether, equal volumes, for 2½ hr.
10. Place specimen in absolute alcohol and ether, equal parts, for 2½ hr.

Embedding:

1. Place the dehydrated bones in 2.5% celloidin on a shaking machine for 3 days.
2. Place in 5% celloidin on a shaking machine for 3 days.
3. Place in 7.5% celloidin on shaking machine for 3 days.
4. Place in 10% celloidin on shaking machine for 3 days.
5. Then embed in fresh 10% celloidin.

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*General Radio Co., Cambridge, Mass.

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Section Two

EXFOLIATIVE CYTOLOGY

HISTORICAL DEVELOPMENT

Exfoliative cytology is the branch of general cytology concerned with the detection of cancer and various other pathologic conditions in man through microscopic examination of body fluids and secreta. Clinical acceptance of exfoliative cytology was long delayed. Early judgments of its usefulness ranged from overly enthusiastic acceptance to emphatic repudiation of the method. Today the exfoliative cytologic technic is a firmly established laboratory procedure, the merits and deficiencies of which are recognized.

The first report of exfoliative cytologic study is credited to Donné,[1] who in 1838 described the morphologic appearances of cells he had observed in human colostrum. In 1843 Walshe[2] reported gross fragments of malignant tissue in sputum. Pouchet[3] in 1847 was the first to describe the cellular morphology of vaginal secretions. Malignant cells in sputum were first described by Beale[4] in 1860 and later in the same year by Hampeln.[5] Similar studies of exudates were reported by Lücke and Klebs[6] in 1867.

Despite the development of improved methods of fixation and staining during the latter part of the nineteenth century and early years of the twentieth century, reports of cellular examinations of body fluids for the detection of cancer were rare. Renewed interest leading to the present clinical role of exfoliative cytology originates from the investigations of Papanicolaou. Prior to 1928 Papanicolaou had examined vaginal secretions from rodents and human subjects in an attempt to correlate the sex cycle with the appearances and patterns of the cells. Using a staining method he had developed to identify stages in the maturation of exfoliated squamous epithelial cells, he was occasionally able to identify malignant cells in human vaginal secretions. In 1928 he published a brief paper[7] advocating use of the vaginal smear method for detection of human uterine cancer. His unusual approach was viewed with skepticism and failed to stimulate confirmatory investigations. Despite the general disinterest, Papanicolaou continued his investigations and in 1943, in collaboration with Traut, published the now famous monograph, *Diagnosis of uterine cancer by the vaginal smear.*[8]

Following confirmation, exfoliative cytology was finally acknowledged as a potentially sensitive and reliable means of detecting early cancer. Soon this method was applied with enthusiasm to the study of sputum, urine, semen, prostatic and breast secretions, aspirations from the bronchus, gastrointestinal tract and cysts, and pleural, peritoneal, and pericardial fluids.

PHYSICIAN-TECHNICIAN TEAM IN CYTODIAGNOSIS

The effective use of exfoliative cytology for the detection of cancer depends on a thorough microscopic examination of every area of an adequate number of slides. A hit-or-miss examination or an over-all impression does not suffice. The prohibitive time required for adequate examination has necessitated training of nonprofessional assistants to screen cytologic smears preliminary to the diagnosis by a physician. Special training is required for both physicians and cytotechnicians, and a number of teaching centers offer such instruction.

The specially trained cytotechnician has demonstrated unusual competence in screening and often assumes responsibility for negative reports. In many cases the cytotechnician has developed considerable skill in identifying morphologic evidences of malignancy. To maintain the clinical validity of exfoliative cytology, it is of utmost importance that the responsibility for a cytologic diagnosis of cancer be restricted to a specially trained and experienced physician who is able to relate morphologic observations and pertinent clinical findings in the individual patient.

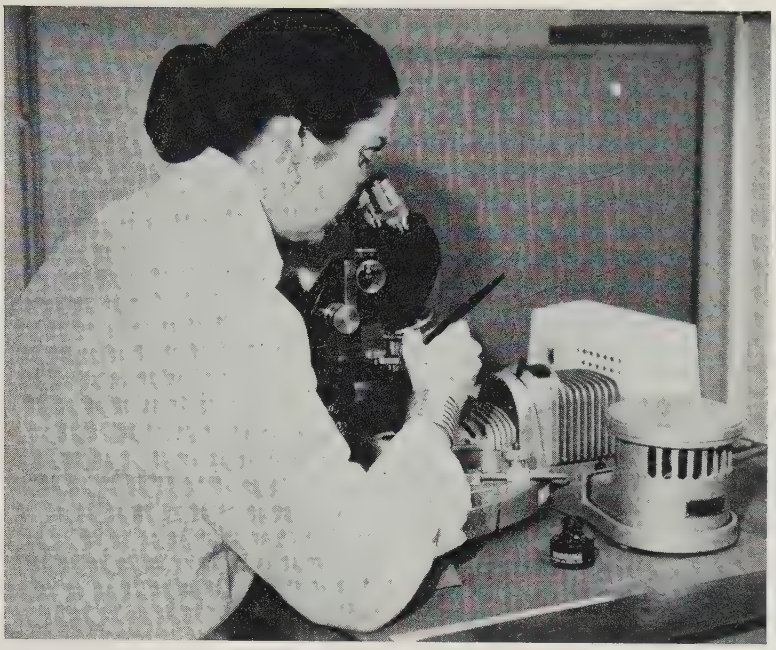

Fig. 584A. Cytologic smears are screened by specially trained cytotechnician who marks cellular abnormalities. Atypical findings are referred to physician for diagnosis.

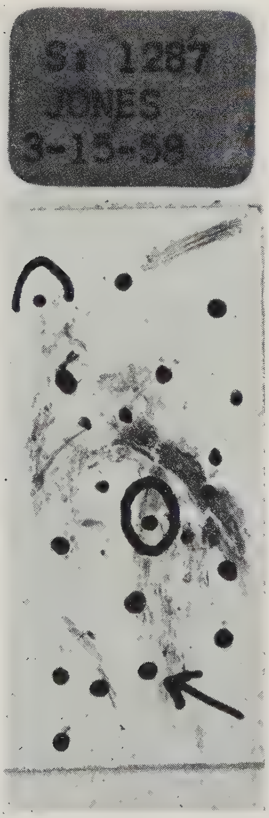

Fig. 584B. Appearance of "positive" smear of sputum following preliminary screening by cytotechnician. Note the use of circle and arrow to call attention to especially important cellular abnormalities.

REPORTING METHODS

Papanicolaou has recommended a method for reporting results of cytologic smear examinations which consists of 5 classes. His method is intended to indicate to the clinician the significance of observed cellular abnormalities as they relate to the possibility of malignancy. The Papanicolaou reporting method is as follows:

Class I—Absence of atypical or abnormal cells.
Class II—Atypical cytology but no evidence of malignancy.
Class III—Cytology suggestive of, but not conclusive for, malignancy.
Class IV—Cytology strongly suggestive of malignancy.
Class V—Cytology conclusive for malignancy.

The reporting of smears that are normal (class I) and those that are malignant (class V) conforms to a relatively uniform standard interpretation by most cytologists; however, reporting of classes II, III, and IV shows wide variations in personal interpretation. Inconsistency in the use of these intermediate classes has made it difficult for one cytologist to evaluate a report made by another worker. In addition, referring physicians often find use of the 5 classes a somewhat elaborate means for answering their question "Is malignancy present or not?" Therefore, to inform the clinician more satisfactorily of the results of cytologic study, many cytologists now use only 3 classes of reports, which indicate (with variations in terminology):

1—"No malignant cells are seen."
2—"Malignant cells are present."
3—"Inconclusive," "Uncertain," or "Opinion deferred."

The latter, inconclusive class combines classes II, III, and IV of the Papanicolaou reporting method and therefore incorporates a wide variety of cellular abnormalities which range from benign atypicalities to those that are frankly suspicious of malignancy.

Successful use of a 3-class method of reporting depends upon establishment of a close rapport between cytologist and referring physician. The cytologists's report should contain a concise statement regarding the significance of findings in each case, with appropriate recommendations for further studies. Whenever possible, any report other than normal (class I), should also be given verbally to the attending physician to promote a clearer understanding.

COLLECTION, PREPARATION, AND STAINING OF CYTOLOGIC SPECIMENS

Successful application of exfoliative cytology to the detection of cancer is directly influenced by the degree of care and attention paid to technical considerations. The method of preparing and processing most of the types of cytologic specimens is often similar; however, the variations in methods best suited for particular specimens deserve special attention.

Since the cytologic identification of exfoliated malignant cells is based upon a morphologic comparison with known normal and benign cellular appearances, it is essential that all cells be visualized in their best possible state of preservation following fixation and staining. Staining reactions must be consistent; otherwise uncertainty may result. An unusual emphasis must be placed upon specimen collection, particularly the specimens that the patient himself collects, such as sputum or urine. When required, the special instructions to the patient for specimen collection must be thorough and explicit to gain understanding and cooperation. Negligent technic in specimen collection and preparation is by far the most frequent basis for failure of the cytologic method to detect evidence of cancer.

Chapter 94

General instructions for cytologic specimens

SAMUEL L. PHARR

An outline of the basic considerations related to the collection, preparation, adhesion, fixation, and staining of cytologic specimens is presented in this chapter. A more detailed account of specific technics for processing individual types of body fluids is given in Chapter 96.

SPECIMEN COLLECTION

The majority of cytologic specimens submitted for diagnosis are collected by an attending physician during the course of a physical examination or as a part of a diagnostic or therapeutic procedure. These specimens include vaginal and cervical smears, bronchial aspiration and lavage, serous cavity fluids, prostatic and breast secretions, and cerebrospinal fluid. Although the attending physician is anxious that the smear examination be a meaningful one, he is often unaware of the critical importance of observing seemingly minor details required for specimen collection. The problem of obtaining properly collected specimens is best resolved through close rapport between the cytologist and the referring physician, preferably through discussion in the laboratory, utilizing microscopic demonstrations of collection errors. Written instructions for the physician's use are at times the only practical solution, but these are limited in effectiveness as compared with verbal and visual means. It is highly desirable that the cytology laboratory establish and adhere to rigid requirements for specimen collection. The desired method of collecting a cytologic specimen is seldom more tedious or time-consuming than an injudicious approach.

Specimens collected by the patient, such as urine and sputum, as well as those specimens that are collected by laboratory personnel, such as gastrointestinal specimens, also have precise requirements. The specific details of these are discussed in Chapter 96.

The importance of correct specimen collection cannot be overemphasized. Failure to disclose malignant cells in cytologic smear preparations, in experienced hands, is seldom attributable to screening error. Tumor cells may fail to shed into the draining secretion because of superficial necrosis or fluid obstruction, or they may be absent because of chance selection of the smeared material. Frequently, however, malignant cells may not be present in smears from patients who have cancer because of some error committed in the method of specimen collection or smear preparation.

SMEAR PREPARATION

Smears prepared for cytologic examination must be made from **fresh** material. New glass slides previously cleaned in alcohol should be used. The patient's identity must be legibly indicated on the slide, preferably with a diamond-point pen, before smears are prepared. Insertion of a paper clip to the identified end of each slide prevents adhesion of slides in the fixative and is also a convenient holder for placing slides into the fixative rapidly.

The method of preparing smears may differ according to the preference of the individual worker or by selection of the method that is best suited for the type of secretion to be examined. There are 4 principal methods of preparing smears for cytologic examination: streaking, spreading, pull-apart, and touch preparation.

Streaking: The method utilizes an applicator stick, gloved finger, wooden spatula, or other means to streak a selected portion of the secretion over ⅔ of a clean glass slide. Care must be taken to streak the material rapidly, attempting to obtain a relatively uniform distribution of the secretion. Smears prepared by this method are more frequently made too thin rather than too thick; either extreme is unsuitable. Streaking is usually employed for preparation of mucoid secretions, such as vaginal secretion aspirated from the posterior fornix, sputum, and gastric contents. Smears must be placed immediately into the fixative before any drying occurs.

Spreading: This method may also be used for preparation of thick, mucoid secretion. A carefully selected portion of the specimen is transferred to a clean glass slide; excess material can be removed by "sawing" with an applicator stick across the sharp glass edge. Holding an applicator stick in each hand, gently spread the material to a moderately thick film by teasing the mucus strands apart. Although spreading is slightly more tedious than streaking, it has the advantage of maintaining cellular interrelationships within strands of mucus. Spreading is especially recommended for preparation of smears of fresh sputum and bronchial aspirates.

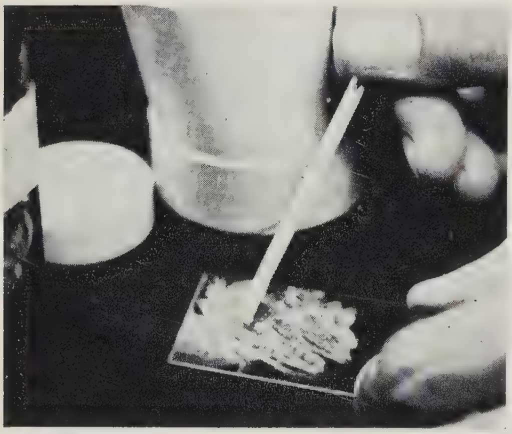

Fig. 585. Cytologic smear preparation by "streaking" method. Note the fixative container is open so that smear can be fixed without delay.

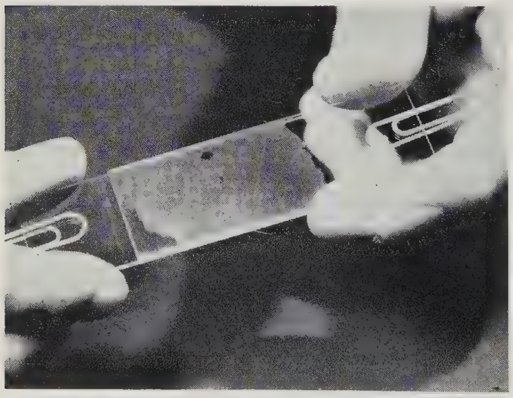

Fig. 586. Smear preparation by pull-apart method.

Pull-apart: Use of this method yields the most desirable smears for cytologic examination because of the resulting uniform distribution of cells. It is applicable, however, only to preparing smears of amucoid secretions, such as serous fluids, concentrated sputum, and enzymatic lavage samples from the gastrointestinal tract. Smears of urinary sediment, vaginal pool, and breast secretions may at times be prepared advantageously by this method.

Two pull-apart smears are prepared simultaneously by placing a drop of secretion or sediment upon one slide and facing it to another clean slide. The material disperses evenly over the surfaces of the 2 slides; slight movement of the 2 slides in opposite directions may be necessary to initiate flow of material. The 2 slides are pulled apart with a single, uninterrupted motion and are then placed immediately into the fixative solution.

Touch preparation: This method is used for the preparation of direct impression smears made from the cut surface of tissues, such as lymph nodes and other surgical or autopsy tissue specimens. The cut surface of freshly incised tissue is touched several times to a clean glass slide **without smearing.** No more than 3 or 4 rapid impressions should be attempted on each slide to avoid drying in thin areas. Each slide is placed immediately into the fixative.

Cytologic smears of scanty secretion or sediment, such as cerebrospinal fluid, buccal smears and others, may be prepared similarly. Scanty material, when spread over a large area of the slide, presents a frequent source of microscopic difficulty due to mechanical distortion and drying. In such cases it is advisable to place a dime-sized circle with a diamond-point pen on the glass surface opposite the side to be smeared. The material collected onto a slightly moistened cotton-tipped applicator stick is touched rapidly within the previously circled area. The circle will aid the microscopist in locating the thin smeared area on the stained slide.

ADHESION

Most body fluids contain sufficient protein in the form of mucus or albumin to provide adequate adhesion of smeared material to the slide throughout fixation and staining. The specimens that require addition of an adhesive agent are urinary sediment, bronchial lavage specimens, and specimens that utilize a proteolytic enzyme during processing, such as tryptic concentrated sputum and enzymatic lavage specimens from the gastrointestinal tract. The adhesive agent used must be permeable to both the fixative and stains and must not retain stain. The latter requirement excludes the use of an egg albumin fixative which is intensely stained by the basic light green counterstain of the Papanicolaou method.

The following methods have been suggested to improve adhesion of material throughout processing for cytologic examination: pooled human serum or plasma, celloidin-ether-alcohol, and leuconostoc culture.

Pooled human serum or plasma

Since the specimens that require addition of an adhesive agent also require centrifugation, use of serum or plasma for this purpose can be easily and effectively employed. Serum or plasma is an effective adhesive agent, and its relatively neutral pH produces no undesirable background staining. Pooled human serum and plasma salvaged from blood chemistry specimens is centrifuged, and the clear supernatant liquid is transferred to small glass bottles in quantities of 2-5 ml. It is stored at freezing temperature until needed. When thawed, it should be discarded after 1 to 2 days.

The method consists in underlaying a portion of liquid serum in the specimen **before** centrifugation. Since it is heavier than the overlying specimen, it forms a separate layer at the bottom of the centrifuge tube. The quantity to be underlaid is determined by the amount of sediment that is expected after centrifugation and is suggested by the turbidity of the specimen. The volume of serum added should be slightly greater than the volume of sediment. During centrifugation the collecting sediment is coated with a film of pooled serum and plasma and serves to produce adhesion of the smeared material during fixation and staining.

An **alternate method** consists in adding a drop of pooled serum and plasma to the surface of one clean slide, a drop of secretion or sediment to another, and then facing the 2 slides. The 2 drops mix and spread over the surfaces of the 2 slides. The slides are pulled apart with an even, uninterrupted motion and are immediately placed in the fixative.

Fig. 587. Serum introduced beneath fluid before centrifugation to improve cellular adhesiveness.

Celloidin-ether-alcohol

Celloidin is readily soluble in a mixture of ether and alcohol but is insoluble in either solution alone; therefore a thin, transparent, and permeable film of celloidin is formed over the surface of smears when transferred from celloidin-ether-alcohol fixative to the alcoholic and aqueous solutions in the Papanicolaou staining procedure. A few drops of chloroform must be added to the absolute alcohol and xylol solutions in the final steps of Papanicolaou staining to prevent fragmentation of the celloidin film in these anhydrous solutions. Celloidin-ether-alcohol solution can be reused after filtration and can be employed routinely for processing all types of cytologic specimens.

The adherent-fixing solution is prepared by adding 1.5 ml. thick celloidin to each liter of equal parts ether and 95% alcohol.

Leuconostoc culture—Rosenthal-Traut[9]

Rosenthal and Traut[9] recommend addition of a nutrient broth culture of *Leuconostoc mesenteroides,* a mucus-producing saphrophyte, to improve adhesiveness of cells in cytologic smears. The culture broth is underlaid in the specimen before centrifugation. It is reported that addition of the liquid culture of *Leuconostoc* to the material to be smeared promotes excellent adhesion of cells. The culture solution does not stain, and the organisms themselves do not interfere with the microscopic examination.

The organisms are cultured at room temperature in equal parts of nutrient broth and 10% sucrose. Inoculated tubes may be kept indefinitely.

FIXATION

Smears prepared for cytologic examination must be immersed in the fixative solution while still moist and before any drying occurs. Structural details of cellular and nuclear morphology are seriously distorted by drying of smear before fixation, and staining reactions lose specificity. Although immediate fixation is required, care must be taken to place each smear in the fixative by a single, uninterrupted motion to avoid rippling of the smeared material. It is also important to avoid striking the bottom of the fixing container forcefully, thus dislodging material from the slide.

Faulty fixation is perhaps the most frequent cause of unsatisfactory cytologic smears. Attention paid to the following points should assist in overcoming this source of technical error:

1. Identify slides **before** preparing smears.
2. Attach a paper clip to the identified end of each slide **before** preparing the smear. Paper clips prevent slides from sticking together in the fixative. Copper or brass paper clips oxidize in ether-alcohol and should not be used.

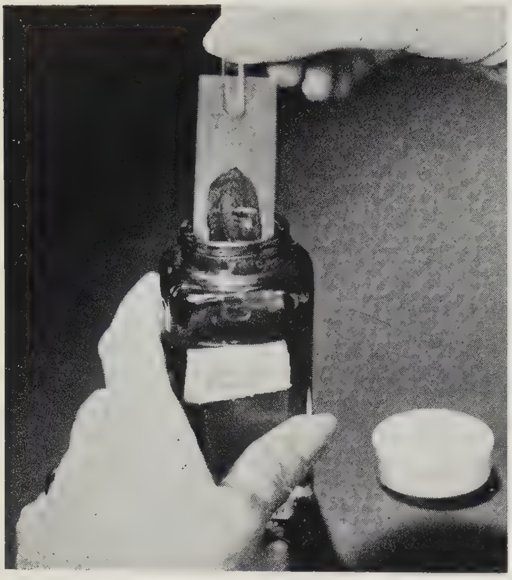

Fig. 588. Cytologic smears must be placed in the fixative immediately after preparation, while still moist. Paper clip is useful as a slide holder and also separates the smears in the fixative.

3. Have the individual fixative bottle open **before** preparing the smear.
4. Do not admire smear after its preparation but place it **immediately** into the fixative.

The minimum fixation time required before staining varies with different specimens. Thin, amucoid smears may be stained after 30 min. fixation; however, it is recommended that all smears be left in the fixative for at least 1 hr. before staining to assure optimum dehydration and adhesion. No untoward consequence results from prolonged fixation; smears may be left in the fixative solution for weeks or months before staining, provided the container is covered tightly to prevent evaporation.

The following solutions may be used for fixation of cytologic smears: ether-alcohol, 95% ethyl alcohol, and Carnoy fluid.

Ether-alcohol: A solution of equal parts of ethyl ether and 95% ethyl alcohol is the conventional solution that is recommended and widely used for fixation of all types of cytologic smears. As previously described, celloidin may also be added to enhance adhesion. The high cost of ethyl alcohol without a tax-free permit may necessitate substitution of methyl or isopropyl alcohol. Ether-alcohol fixative solution may be filtered and reused.

95% Ethyl alcohol: Fixation of cytologic smears in 95% ethyl alcohol has been advocated, substituting methyl or isopropyl alcohol whenever necessary. The omission of ether does not appear to alter the quality or effectiveness of fixation and eliminates the hazard of working with highly flammable ether.

Carnoy fluid:

Alcohol, absolute	6 parts
Chloroform	3 parts
Glacial acetic acid	1 part

Rapid penetration by anhydrous Carnoy fluid allows staining of smears after 5 min. fixation. The solution is more expensive than ether-alcohol or alcohol fixative solutions and cannot be reused effectively. Although Carnoy fluid is not usually suitable for routine usage, it is useful in a number of cytologic research applications and is particularly valuable when rapid fixation and staining are required. It has been recommended for use in membrane filtrate methods.

SMEARS REFERRED ELSEWHERE FOR STAINING

It is often necessary for physicians to mail cytologic smears elsewhere for staining and examination. Since postal regulations forbid mailing flammable solutions, it is not possible to mail smears in the ether-alcohol or alcohol fixative. A number of methods have been suggested to minimize the effect of drying of smears in preparation for mailing.

Air-dried-fixed smears

Many workers find that slides, after adequate fixation in ether-alcohol at the time of preparation, may be removed from the fixative, dried in the air, and mailed without further processing. The simplicity of this method is appealing to the referring physician, and its use undoubtedly encourages greater clinical utilization of the smear technic for cancer detection. The most consequential cellular alterations result from the effect of smear drying **before** fixation, and the changes resulting from smear drying after adequate fixation are undesirable but much less significant.

Glycerin technic

Ayre and Dakin[10] recommend that ether-alcohol-fixed slides be allowed to dry in the air and then be covered with glycerin. The glycerin-coated smear is then placed face to face with another clean slide or with another air-dried, fixed smear from the same specimen. The principal objection to this method has been the loss of considerable material when the 2 faced slides are separated.

Diaphane technic*

A method reported by Papanicolaou and Bridges[11] suggests flooding freshly prepared, unfixed, wet smears with an alcoholic solution of Diaphane.

Diaphane solution[11]:

Ethyl alcohol, 95%	3 parts
Diaphane*	3 parts

After drying, a hard, smooth film which protects the smear surface from contact with the air is formed. When received in the cytology laboratory, smears are placed in a solution of equal parts of ether and 95% ethyl alcohol for 20 min. to remove the Diaphane. Routine Papanicolaou staining follows without modification.

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*Diaphane is a synthetic resin made by the Will Corporation, Rochester, N. Y.

Polyethylene glycol (Carbowax*) technic

Sills[12] first described a method of dipping ether-alcohol-fixed smears, while still wet, into melted polyethylene glycol prior to mailing. Later, Sills and Garret[13] modified the method whereby 5% polyethylene glycol was added directly to the ether-alcohol fixative. Freshly prepared smears placed in this solution for 30-60 min. and then removed and dried for mailing are thus fixed and coated with a protective film in a single step.

Ehrenreich and Kerpe[14] have reported that equally good results can be obtained more simply by merely flooding freshly made smears, placed on a flat surface, with a 5% solution of polyethylene glycol in ether-alcohol. The slides are allowed to evaporate to dryness. After 5-7 min. an opaque, waxy film is formed and slides can be packaged and mailed.

Polyethylene glycol is water-soluble and does not need to be removed prior to staining by the Papanicolaou method. Smears fixed in this manner are placed directly in aqueous hematoxylin without previous treatment.

Polyethylene glycol fixing solution:

Ethyl alcohol, 95%	50 ml.
Ether	50 ml.
Polyethylene glycol	5 gm.

Polyethylene glycol must be softened in a 56° C. incubator before it is added to the ether-alcohol mixture. A clear, stable solution is obtained after standing for several hours. Dissolution may be hastened by agitation.

Richardson glycol fixative[15]

Richardson has reported a nonvolatile, noncombustible, and nonexplosive fixative for cytologic smears which can then be mailed in conformance with postal regulations. The fixative is reported to have the additional advantage of clearing mucus and hemolyzing erythrocytes. The solution is inexpensive and can be reused.

Richardson glycol fixative:

Monoethylene glycol	3446 ml.
Diethylene glycol	181 ml.
Borax pentahydrate	36 gm.
Water	5776 ml.
Glacial acetic acid	500 ml.

Rehydration technic—Nieburgs[16, 17]

Nieburgs has developed a rehydrating procedure which eliminates fluid fixation at the time of smear preparation. The method was originally designed to improve the quality of self-obtained vaginal smears in a mass survey.

Reagents:

1. Aqueous rehydrating solution.
   (a) Dissolve 0.5 ml. polyoxyethylene sorbitan monoaleate* in 1000 ml. heated distilled water. Allow to cool before use.
2. Ether-alcohol solution.
   (a) Prepare a mixture of equal parts of ether and 70% alcohol.
3. Rehydrating fluid.

Aqueous rehydrating solution	250 ml.
Ether-alcohol solution	1000 ml.

Procedure: Cytologic smears that are to be sent elsewhere for staining and examination are prepared in the usual manner; however, they are not placed in a fixative but are allowed to dry in the air. Afterward they may be packaged and mailed.

Before staining, smears are immersed for 1 min. in the rehydrating fluid.

Slides are rinsed gently in a solution of equal parts of 95% alcohol and ether and are then transferred to a second ether-alcohol rinse, where they remain for 10 min.

NOTE: Following rehydration, small amounts of the rehydrating fluid may be carried into the staining solutions, thereby increasing cellular absorption of stains. The author therefore suggests that staining periods be considerably reduced when rehydrating fluid is used.[17]

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*Polyethylene glycol is available as Carbowax Compound 1540 from Union Carbide Chemicals Co., Division of Union Carbide Co., New York, N. Y.

*Polyoxyethylene sorbitan monoaleate available as Tween 80, Atlas Powder Co., Wilmington, Del.

Chapter 95

Cytologic staining methods

SAMUEL L. PHARR

PAPANICOLAOU METHOD

The compound staining procedure developed by Papanicolaou[18] has for nearly 2 decades remained the staining method of choice by most cytologists. The principal advantage of Papanicolaou's staining method is the transparency obtained by use of counterstains of high alcoholic content. The color range is predictable and is of great assistance in identification and classification of cells.

Since its description in 1942, Papanicolaou has made only minor changes in the original counterstain formulas and in staining times. The Papanicolaou staining procedure that follows is from the 1960 Supplement to his *Atlas of exfoliative cytology.*[19]

Preparation of Papanicolaou stains[19] * †

Harris hematoxylin:

Stock Harris hematoxylin:

Hematoxylin	5 gm.
Ammonium aluminum sulfate	100 gm.
Ethyl alcohol, 95%	50 ml.
Distilled water	1000 ml.
Mercuric oxide	2.5 gm.

1. Dissolve the hematoxylin in the alcohol.
2. Add the ammonium aluminum sulfate to the water and heat to the boiling point.
3. Add the hematoxylin solution and bring to a full boil once more.
4. Remove from the heat and add the mercuric oxide at once. Stir quickly until a black-purple color develops—a matter of seconds.
5. Plunge the container into cold water.
6. When cool, filter into a dark bottle.

The stock stain must be aged at least 2 wk. before use.

Harris hematoxylin staining solution:

Aged stock Harris hematoxylin	100 ml.
Glacial acetic acid	4 ml.

OG-6 counterstain, stock:

1. Prepare a 10% stock solution of orange G in distilled water.

OG-6 staining solution:

Ethyl alcohol, 95%	950 ml.
Aqueous stock OG solution, 10%	50 ml.
Phosphotungstic acid	0.15 gm.

EA-65 counterstain, stock:

1. First prepare 10% solutions of light green SF yellowish, Bismarck brown, and eosin Y, water- and alcohol-soluble, in distilled water.
2. Next, prepare 3 **alcoholic stock solutions** from the aqueous stock solutions as follows:
   A—Light green SF yellowish: 0.05% solution in 95% ethyl alcohol
   B—Bismarck brown: 0.5% solution in 95% ethyl alcohol
   C—Eosin yellowish: 0.5% solution in 95% ethyl alcohol

EA-65 staining solution:

Alcoholic stock solution A	180 ml.
Alcoholic stock solution B	40 ml.
Alcoholic stock solution C	180 ml.
Phosphotungstic acid	2.4 gm.

1. Filter and store in a dark bottle.

Papanicolaou staining procedure[19]

1. Carry out staining procedure in Coplin jars or staining dishes.
2. After fixation, transfer slides, without drying, directly from the ether-alcohol mixture to 80% alcohol and pass through 70% and 50% alcohols to distilled water.
3. Stain in Harris hematoxylin staining solution for exactly 45 sec.

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*Stains prepared by the Papanicolaou formulas are obtainable from the Gradwohl Laboratory Supply Co., St. Louis, Mo., and from Ortho Pharmaceutical Corp., Raritan, N. J.

†All dyes used in these preparations are certified stains supplied by National Aniline Division, Allied Chemical & Dye Corp., New York, N. Y.

4. Rinse 3 times in distilled water, using 3 separate containers. Rinsing should be gentle to prevent loss of material from slides.
5. Rinse in 50% alcohol.
6. Place in a solution of 1.5% ammonium hydroxide in 70% alcohol for 1 min.
7. Rinse in 70% alcohol and pass through 80% and 95% alcohols.
8. Stain in OG-6 staining solution for 1¼ min.
9. Rinse in 3 changes of 95% alcohol.
10. Stain in EA-65 staining solution for 3 min.
11. Rinse in 3 changes of 95% alcohol.
12. Dehydrate and clear by passing through the following:
    (a) absolute alcohol
    (b) a mixture of equal parts of absolute alcohol and xylol
    (c) xylol
13. Mount.

MODIFIED PAPANICOLAOU STAINING PROCEDURE—PHARR, WOOD, AND TRAUT[20]

The original Papanicolaou method of staining and stain preparation has been slightly modified by Pharr et al.[20] The formulas of the Papanicolaou counterstains were changed to produce a more distinct tinctorial differentiation between eosinophilic and orangeophilic cytoplasmic staining. This is accomplished by increasing the amount of phosphotungstic acid and omitting Bismarck brown dye from the EA formula. Improved sharpness of color and brilliance of staining reactions result from preparation of counterstains directly in alcohol rather than from aqueous stock solutions. Use of a mechanical stirrer such as a Waring blendor facilitates dissolution of the powdered stains in alcohol.

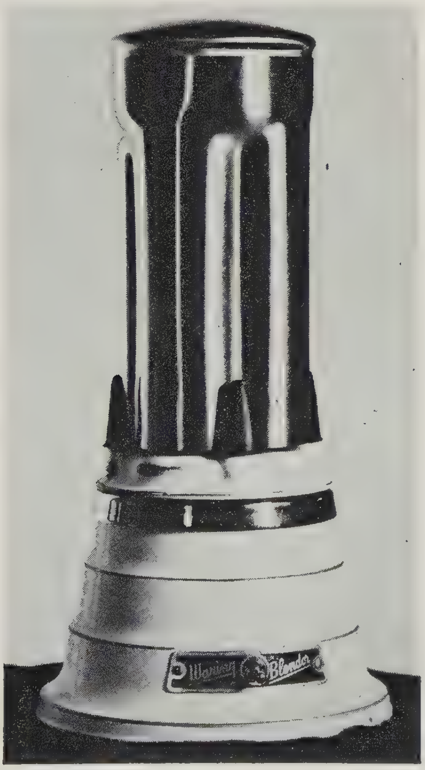

Fig. 589. Mechanical stirrer used for dissolution of Papanicolaou counterstains in alcohol.

Preparation of counterstains—Pharr, Wood, and Traut modification[20] * †

Orange G:

1. Heat 380 ml. 95% ethyl alcohol to the boiling point and pour sufficient heated alcohol into the mechanical stirrer cup to cover the blades.
2. Add 1 gm. orange G.*
3. Fill the stirrer cup with heated alcohol to approximately ¼ its volume.
4. Mix the solution for 5 min., turning the motor off and on occasionally to obtain maximum mixing.
5. Pour off the supernatant liquid after allowing unsuspended stain to settle momentarily.
6. Add additional alcohol to the remaining undissolved stain and mix as before.
7. Repeat until all alcohol has been used and all stain is in solution.
8. Allow the solution to **cool** and then filter. There will be a slight precipitate of orange G stain after cooling.
9. Add 0.4 gm. phosphotungstic acid and 3 ml. glacial acetic acid to the filtered solution.

The orange G staining solution is then ready for use and is stable for 1 mo.-6 wk. Store excess staining solution in a dark bottle.

EA staining solution:

1. Cover the blades of a clean, **dry** mechanical stirrer with a portion of a measured 380 ml. 95% ethyl alcohol. Do not heat the alcohol.
2. Add 2 gm. eosin Y and 1 gm. light green SF yellowish and fill the stirrer cup to approximately ¼ its volume with alcohol. Note that Bismarck brown has been omitted as a constituent of this stain.
3. Mix, as previously described, until all the stain is in solution.
4. Allow to stand overnight, but **do not filter.**
5. Add 0.4 gm. phosphotungstic acid and 3 ml. glacial acetic acid.

The EA staining solution is stable for 1 mo.-6 wk. if it is covered to protect it from deterioration by light or poured back into a dark bottle each time after use.

Stains prepared in this manner have been

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*All stains used in these preparations are certified stains prepared by National Aniline Division, Allied Chemical Corp., New York, N. Y.

†Papanicolaou counterstains prepared by the Pharr, Wood, and Traut formulas are obtainable from the Gradwohl Laboratory Supply Co., St. Louis, Mo.

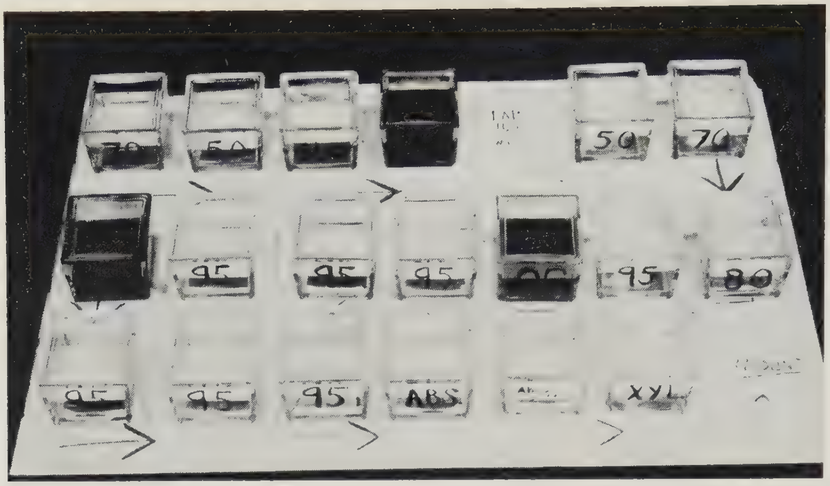

Fig. 590. Arrangement of staining dishes for Papanicolaou staining method.

consistent from one batch to another and have not precipitated during use.

Mayer hematoxylin:

Distilled water	1000	ml.
Hematoxylin	1	gm.
Ammonium or potassium alum	50	gm.
Sodium iodate	0.2	gm.
Citric acid, crystalline	1	gm.
Chloral hydrate	50	gm.

1. Dissolve the hematoxylin in the distilled water that has been heated to 55°-60° C. and rotate until all the stain is in solution.
2. Add the alum and sodium iodate, taking particular care that exactly 0.2 gm. sodium iodate has been weighed.
3. Add the citric acid next, followed by chloral hydrate. **The order of mixing must not be changed.**
4. Allow the solution to stand overnight before use.

The solution is stable for 6 wk.-2 mo. if the staining time is gradually increased from an initial 6 min. to 8 min. after prolonged use.

Occasional filtering may be necessary to remove accumulated cellular debris.

Staining procedure—Pharr, Wood, and Traut modified stains

1. Prepare slides and fix immediately in equal parts of ether and 95% ethyl alcohol for a minimum of 30 min.
2. Immerse in 70% ethyl alcohol for approximately 10 dips or until glossy.
3. Immerse in 50% ethyl alcohol for approximately 10 dips or until glossy.
4. Rinse in **distilled** water for approximately 10 dips or until glossy. This water should be changed after each use.
5. Stain with Mayer hematoxylin for 6-8 min., according to the age of the stain.
6. Wash in a gentle stream of running tap water for a minimum of 20 min.
7. Dehydrate in graded ethyl alcohol, 50%, 70%, 80%, and 95%, immersing for 10 dips in each.
8. Stain in orange G staining solution 1½ min., agitating occasionally.
9. Immerse for 10 dips each in 3 changes of 95% ethyl alcohol; the third 95% alcohol rinse solution should always be clear.
10. Stain in EA staining solution 1½ min., agitating occasionally.
11. Immerse for 10 dips each in 3 changes of 95% ethyl alcohol; the third 95% alcohol rinse solution should always be clear.
12. Dehydrate in absolute ethyl alcohol, immersing for 10 dips.
13. Immerse in a mixture of equal parts of absolute ethyl alcohol and toluene, 10 dips.
14. Clear in 2 changes of toluene, immersing for approximately 10 dips in each.
15. Mount in rapid drying mounting medium.*

Note: If the local tap water used in washing slides after staining by Mayer hematoxylin is not sufficiently alkaline, i.e., approximately pH 7.4, the lithium carbonate method for alkalinizing must be employed.

Prolonged standing in the alcohol rinses following staining with EA results in excess decolorization of both eosin and light green. The stained slides may stand in toluene for a short period but no longer than 1-2 hr.

Prevent water absorption and evaporation by keeping the staining dishes covered when not in use.

Staining tissue sections by modified Papanicolaou method

Tissue sections stained by the Papanicolaou method are of value in comparing cellular appearances in smears with their counterparts in similarly stained sections. The method is used primarily for preparation of material in cytology teaching centers.

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*Rapid drying mounting medium available as Eukitt from O. Kindler, Konradstrasse No. 25, 1 Br. Freiburg, Germany.

Procedure:

1. Remove paraffin in 2 changes of xylol, 5 min. in each.
2. Rinse in 2 changes of absolute ethyl alcohol, immersing 10 dips in each.
3. Immerse in 95% ethyl alcohol for approximately 10 dips.
4. Immerse in 50% ethyl alcohol for approximately 10 dips.
5. Rinse in distilled water for approximately 10 dips.
6. Stain with Mayer hematoxylin for 15 min. Note that the staining time is increased.
7. Continue with previously described staining procedure (p. 1766). The staining times in orange G and EA remain the same.

Note: It is preferable to stain paraffin sections by this method on the same day they are cut. Do not place cut sections on a heated drying rack before staining.

ADDITIONAL CYTOLOGIC STAINING METHODS

Other staining methods in addition to the Papanicolaou procedure have been recommended for cytologic examination. The standard hematoxylin and eosin method is preferred by many because of familiarity with its use in histologic preparations.

Polychrome method—Shorr[21]

The polychrome staining method described by Shorr for cytologic smears is rapid and to some extent differential. The Shorr method has a limited following in this country[22] but is more widely used by European investigators.

Shorr stain[23]:

Ethyl alcohol, 95% . . .	100	ml.
Biebrich scarlet, water-soluble	0.3	gm.
Orange G	0.1	gm.
Aniline blue, water-soluble	0.025	gm.
Fast green FCF	0.075	gm.
Phosphotungstic acid, c.p.	0.25	gm.
Phosphomolybdic acid, c.p.	0.25	gm.
Glacial acetic acid . . .	1	gm.

Procedure:

1. Following fixation in equal parts of ether and 95% ethyl alcohol, immerse slides in Shorr stain for 2 min.
2. Drain off any excess stain and rinse by dipping 10 times each into 80%, 95%, and absolute alcohol.
3. Clear in xylol and mount.

Cresyl violet method—Masin-Masin[24]

Masin and Masin[24] recommend cresyl violet staining in exfoliative gynecologic cytology and stress the simplicity and rapidity of the technic and its resulting clarity of morphologic cellular details.

Cresyl violet stain:

Cresyl violet (National aniline)	0.3	gm.
0.01M Acetate buffer, pH 4.0	100	ml.

Filter each time before use.

Procedure:

1. Allow ether-alcohol fixed slides to dry in the air. These may be kept for several months before staining.
2. Hydrate through 70% and 50% alcohol and distilled water.
3. Rinse in 1% acetic acid for 30 sec., agitating gently.
4. Rinse in 2 changes of distilled water.
5. Stain in freshly filtered cresyl violet staining solution for 3 min., agitating gently during the first 15-30 sec.
6. Drain and dehydrate briefly in 2 changes of acetone, 10 and 5 sec., respectively.
7. Clear in 2 changes of xylol, 5 min. each, and mount.

Additional staining methods that have been applied to cytologic specimens include (1) supravital staining by a wet film method,[25, 26] (2) the periodic acid–Schiff–PAS staining procedure,[27, 28] (3) silver stain method,[29] (4) the May-Grünwald Giemsa procedure,[30] and (5) the Feulgen reaction.[31]

ADDITIONAL MICROSCOPIC TECHNICS

Cytologic examination by microscopic technics other than the conventional light microscope have been introduced and have provided interesting and provocative findings. At present it appears that these technics may supplement rather than substitute for the routine Papanicolaou method.

Acridine orange fluorescence technic

Acridine orange dye, a basic fluorochrome, binds nucleic acids, and when the stained preparation is irradiated with ultraviolet light in the fluorescence microscope, 2 kinds of nucleic acids, deoxyribonucleic (DNA) and ribonucleic (RNA) acids, fluoresce at different wavelengths.

Acridine orange-stained fixed smears studied with the aid of the fluorescence microscope have recently been used as a screening technic in exfoliative cytology.[32-38] The time required for microscopic screening is reported to be considerably less than that needed to examine

Papanicolaou-stained smears. Acridine orange-stained smears can be restained by the Papanicolaou method when indicated.

The basic fluorochrome, acridine orange, differentiates RNA in the cytoplasm and nucleolus in shades ranging from brick to orange and red, and DNA in the nucleus is stained in shades of green and yellow. Increased basophilia (increased RNA concentration) is an expression of growth per se and is not a specific expression of malignancy. Proliferating benign cells often have the same degree of cytoplasmic basophilia as do malignant proliferating cells. It should be emphasized that degenerating or acidophilic **malignant** cells stain green, a further exception to a specific color reaction of malignant cells stained by the acridine orange fluorescence method.

Acridine orange staining—Masin-Masin[32]

Reagents:

1. Acridine orange stock solution.
    - Acridine orange . . . . 0.1 gm.
    - Distilled water . . . . . 100 ml.
    - (a) Store in a dark bottle and refrigerate.
2. Acridine orange staining solution.
    - Acridine orange stock solution . . . . 10 ml.
    - M/15 Phosphate buffer . . . 100 ml.
3. M/15 Phosphate buffer.
    - Sodium phosphate dibasic . . . . . . 10.4104 gm.
    - Sodium phosphate monobasic . . . . . 84.88 gm.
    - Distilled water . . . . 10 L.
    - (a) If the pH deviates from the required 6.0, correct by adding basic or acid component. The buffer is stable indefinitely at room temperature.
4. 0.1M Calcium chloride solution.
    - Anhydrous calcium chloride . . . . . . 11.1 gm.
    - Distilled water . . . . . 1000 ml.

Procedure:

1. Ether-alcohol-fixed smears can be air-dried and stored for several months before staining, if required.
2. Hydrate smears by passage through 80%, 70%, and 50% alcohols and distilled water for approximately 10 dips in each.
3. Immerse in 1% acetic acid for 5 dips.
4. Rinse in 2 changes of distilled water for approximately 10 dips each.
5. Immerse in acridine orange staining solution for 3 min.
6. Wash with a gentle stream of M/15 phosphate buffer from a plastic washing bottle for 1 min.
7. Wash with a gentle stream of 0.1M calcium chloride solution from a plastic washing bottle for 1 min.
8. Repeat M/15 phosphate buffer wash as described.
9. Repeat 0.1M calcium chloride wash as described.
10. Repeat M/15 phosphate buffer wash again.
11. Mount in M/15 phosphate buffer with a cover slip.

Note: All glassware used in the acridine orange staining procedure must be cleaned carefully and rinsed in alcohol or acetone to remove any absorbed stain.

Introduction of the acridine orange fluorescence technic for cancer detection has stimulated a controversial reception which is remarkably parallel to the early emphatic and opposing opinions expressed of the Papanicolaou smear method and is an indication of the potential importance of the fluorescence method. It appears that a cautious attitude should be adopted for the present pending more definitive investigations of the exact value, convenience, and reliability of the acridine orange fluorescence method.

Phase contrast microscopy

Wied[39-41] has made an extensive comparative evaluation of phase microscopy and the routine Papanicolaou method. He concludes that phase microscopy is the "second best choice" for routine cytologic examination. He cites the possibility for immediate microscopic examination as the major advantage and suggests utilization of phase microscopy as an additional technic employed in cancer detection and for hormonal evaluation of gynecologic specimens.

Interference microscopy

The principle of interference microscopy stems from the fact that a beam of light passing through a dense medium or object undergoes a retardation of its phase as compared with a reference beam.[42] Thus it is possible by means of interference microscopy to determine the dry weight of individual cells or cellular constituents. Hirst[43] has described interference microscopy as "phase microscopy in color." Although there has been some evidence that the per cent dry-weight content of the carcinoma cell nucleus and cytoplasm is less than that of the normal cell as indicated by Mellors[44] and also by Foraker,[45] the cost and complexity of interference microscopy preclude its use for routine examination.

Chapter 96

Cytologic examination of secreta from various sources

SAMUEL L. PHARR

The basic considerations related to technics for collection, preparation, and staining of cytologic specimens have been discussed. The reader is urged to familiarize himself with Chapter 95 before proceeding with specific methods for collecting and processing individual types of specimens.

GYNECOLOGIC CYTOLOGY

Smears obtained from the female genital tract greatly predominate among the types of specimens submitted for routine cytologic examination. A wide variety of methods can be applied; however, for best results selection of method of collection and preparation should conform to the recommendations made by the cytology laboratory to which the smears are to be referred. Selection of collection method may depend upon the purpose of the cytologic examination, whether for cancer detection or for evaluation of hormonal and inflammatory changes. The choice of method may also depend upon whether the sample is to be collected by a physician or, as in mass screening programs, by nonprofessional assistants, or even by the patient herself.

Preparation of patient

It is essential that the patient be advised not to douche, bathe, or use intravaginal medicaments (including contraceptives) for a 24 hr. period prior to specimen collection. At least 6 wk. should elapse following cauterization, curettage, or biopsy.[46] The speculum, when inserted before smear collection, must be introduced without lubricant; if necessary, a few drops of warm saline may be used to moisten the speculum.

Vaginal smear

Collection of pooled secretions from the posterior vaginal fornix, as originally described by Papanicolaou and Traut,[47] has remained the most widely used single method for cytologic detection of uterine cancer. Most authorities today, however, insist that both a vaginal pool smear as well as a cervical smear be collected from each patient. The vaginal pool smear should be collected through a speculum whenever possible and practical.

Methods of collecting vaginal smears

Pipet: A specially designed, slightly curved, blunt-end, glass pipet fitted with a rubber bulb is inserted, with negative bulb pressure, about 3-4 in. into the vagina. Secretion is aspirated gently for ½ min., moving the end of the pipet from side to side to assure collection of a good sampling of cells. A portion of the aspirate is placed on a clean glass slide bearing the patient's identity. The material is smeared and the slide is immediately placed in the fixative while still moist.

Spatula: Vaginal secretion may be obtained through a speculum by use of the special wooden spatula described by Ayre[48]* (see below) or by a common tongue blade. The smear is prepared by applying the material collected on the spatula directly to the slide. Immediate fixation, before any drying occurs, is essential.

Speculum blade: The secretion present on the posterior blade of a bivalve speculum after withdrawal may be used for the vaginal smear preparation. The gloved fifth finger (not covered by glove powder) is dipped into the secretion and is then

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*Clay Adams, Inc., New York, N. Y.

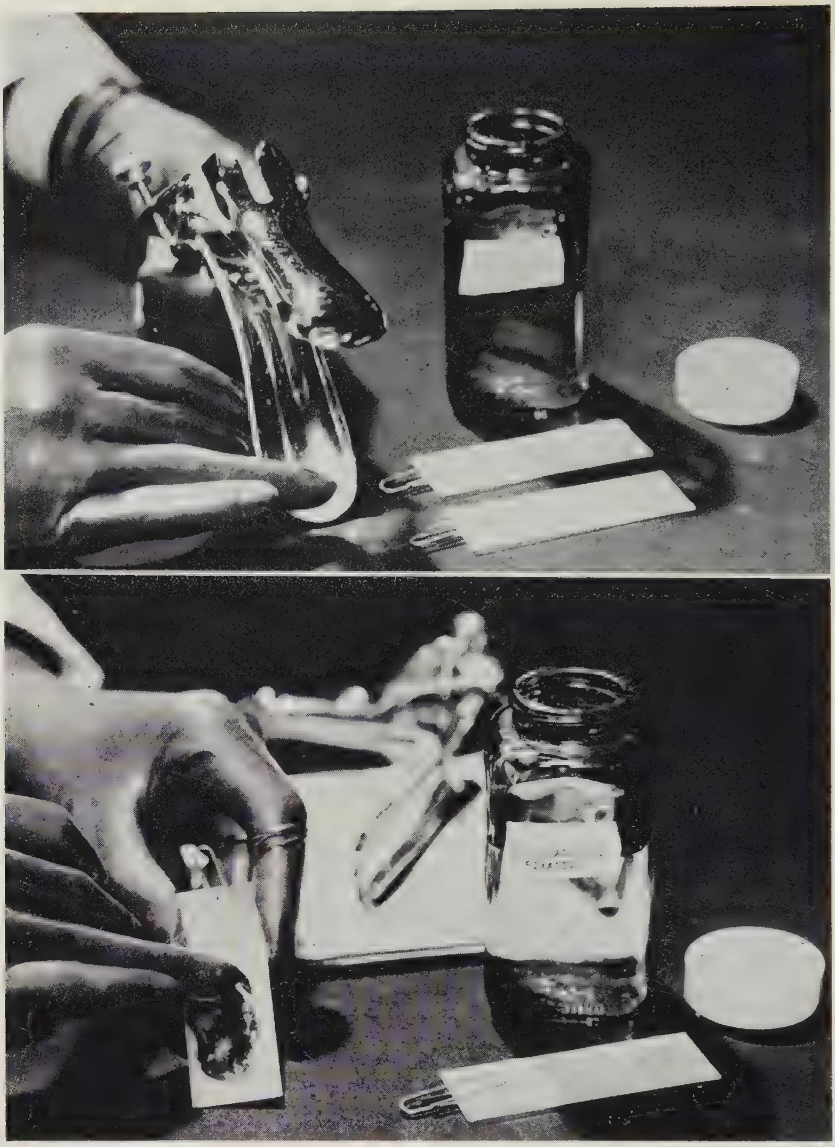

Fig. 591. Vaginal pool secretions collected by clean gloved finger (above) and quickly spread over slide (below).

quickly streaked lengthwise upon a clean glass slide, making 2 parallel strokes.[49] Fix smears immediately.

Cotton swab: An applicator stick tipped with nonabsorbent cotton may be used to obtain vaginal secretions from the posterior fornix through the speculum. The method has also been advocated as a method acceptable to the patient for self-obtained vaginal smear collection.[50]

Cervical smear

Collection of cellular material directly from the cervix increases the detection rate of early cervical cancer, but it is of very little value for detection of fundal carcinoma.[51] According to von Haam,[52] the most satisfactory method to date for cytologic examination of the female genital tract is accomplished by use of the vaginal smear and the cervical smear **together,** taken by a physician after visualization of the cervix by speculum.

Collection of cervical smears

Ayre spatula*: The cervical os is scraped 360° with a specially designed wooden spatula described by Ayre or by means of a common tongue blade that has been cut with curved scissors to fit the contour of the cervix.[53] It is important that the scraper fit the external os, which serves as a pivot for rotation. Collected material is rapidly streaked from the spatula onto a slide and immediately fixed.

Cotton swab: The entire portio vaginalis of the cervix is swabbed with a cotton-tipped applicator stick, introducing the applicator as far as possible into the external os. Material obtained in this manner is best smeared by a rapid rolling motion of the applicator tip on the slide. Murray[54] recommends use of cotton-tipped applicator sticks coated with collodion to produce slight abrasion during collection.

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*Clay Adams, Inc., New York, N. Y.

Direct cervical imprint: A new approach reported by Nieburgs[53] and also by Nyklicek[55] employs a specially designed slide and slide holder whereby a direct impression smear of the cervix may be made, which maintains anatomic relationships and thus may assist in locating occult lesions.

Brunschwig-Draghi tampon: Under ideal circumstances smears prepared with the Draghi tampon* may be considered partially of cervical origin. The method of collection described by Brunschwig[56] and favorably evaluated by Papanicolaou[57] as a mass screening technic utilizes a specially designed tampon encased in a sheath of nonabsorbent nylon.

The tampon is inserted into the vagina by the patient through a cylindrical cardboard container with a narrower cardboard plunger. The tampon is removed after 5-30 min. and smears are prepared by quickly dabbing the distal end of the tampon upon the surface of a clean glass slide. The slide is placed immediately in the fixative. Papanicolaou[57] reported that smears of better quality are obtained when the tampon has remained inserted overnight.

Nieburgs[58] has modified the Brunschwig-Draghi method for use in mass screening whereby the patient also prepares the smears by pulling the withdrawn tampon through a box device† containing 2 slides. Two smears are automatically prepared. The smears are rehydrated before staining and need not be fixed at the time of preparation.

Endocervical smear: Much interest has been directed to collection of endocervical material and particularly to collection of the mucus plug from the external cervical os. It is believed that this material serves as an ideal reservoir for malignant cells when cervical cancer is present. The material may be conveniently and satisfactorily collected with a cotton applicator slightly moistened with physiologic saline, or a pipet or metal cannula may be used. Collection of the cervical mucus plug or endocervical aspiration should precede cervical scraping.

Combined cervicovaginal smear: Several methods have been reported which combine upon a single slide material originating from multiple female genital sites. Frost[59] has described a rapid and economic method which he reports to be suitable for routine usage. A drop of secretion obtained from the posterior vaginal fornix is mixed **on the slide** with material obtained by circumferential cervical scraping.

Wied[60] insists that collection of smears from 3 sites (vaginal pool, cervix, and endocervix) is required from each patient for complete and efficient evaluation. Wied and Bahr[61] reported a method whereby these 3 secretions are separately smeared upon a single, specially designed, partitioned slide—the **VCE slide.*** Specimens are not smeared until all 3 secretions have been obtained. The vaginal and cervical material remains as a thick drop on the collecting instrument, not subject to drying, until the scant endocervical material has been collected and smeared. Vaginal and cervical secretions are then rapidly smeared in the appropriate divided areas of the VCE slide. Speed in smear preparation and immediate fixation are essential. Wied introduced the 3-smears-on-1-slide method as a routine procedure at the Chicago Lying-In Hospital 5 yr. ago and reports that the method has achieved excellent acceptance and utilization by the attending staff.[62]

Intrauterine smears

Cytologic detection of fundal carcinoma is improved through examination of secretions obtained directly from the uterine cavity. The procedure is attended by definite risks, however, and present methods for collection of endometrial secretion do not permit routine usage. Cytologic examination of intrauterine smears should not be considered a substitute for curettage. This type of examination must never be performed during pregnancy. Endometrial cells are well preserved in smear preparations made directly from the uterine cavity in sharp contrast with their often degenerative appearances in cervical and vaginal smears.

Rakoff[63] stated in 1960 that his accuracy in detecting cases of endometrial cancer improved from 66 to 92% by including endometrial aspiration smears in addition to cervical and vaginal smears in selected cases. In a series of 63 patients with uterine carcinoma, Cuyler et al.[64] examined endometrial aspiration smears and were able to diagnose 84.1% as positive or suspicious.

Reports of additional intrauterine smear examinations have been made by Hect[65] and by Reagan and Sommerville.[66]

Endometrial aspiration: A number of instruments have been designed for collection of secretion from the endometrium. These include various types of metal cannulas, polyethylene tubing, and a number of abrasive devices. For specifications and sources of instruments see Nieburgs,[67] and for full details of intrauterine technics see Romberg.[68]

Endometrial lavage: Morton et al.[69] described a lavage method in 1957 for obtaining representative samples from the endometrial cavity. A small quantity of sterile saline is injected into the uterine cavity and the return flow is then aspirated from the posterior vaginal fornix.

In a series of 797 lavages obtained from 673 patients, the investigators accurately diagnosed uterine cancer in 24 of 26 (92%) histologically proved cases. In 11 cases, however, the authors erroneously interpreted benign endometrial cells as suspicious or positive.

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*Available as Draghi tampon from the Tampax Corp., Palmer, Mass.

†Available as Nieburgs cancer detection tampon with automatic smear preparation device from Personal Products Corp., Milltown, N. Y.

*Available as VCE cytologic slides from Glasco Hospital Products, Chicago, Ill.

PULMONARY CYTOLOGY

The introduction of the Papanicolaou smear technic in 1943[70] for cancer diagnosis coincided closely with the development of serious concern among clinicians over the increasing incidence of lung cancer. There was no delay in investigating the reliability of cytologic examination of sputum and bronchial secretions for lung cancer detection. Wandall,[71] in a report published in 1944, was able to identify malignant cells in the sputum of 84 of 100 patients with primary carcinoma of the lung. In 1946 Herbut and Clerf[72] accurately diagnosed 47 of 57 cases (82%) of carcinoma of the lung by examining bronchoscopically obtained secretions. Farber et al.[73] in 1948 reported an accuracy of 80% and emphasized the necessity of examining multiple specimens from each case. Additional studies reported by Woolner and McDonald,[74] Liebow et al.,[75] and by McKay et al.[76] were in close agreement with the previously reported accuracy and sensitivity of pulmonary cytology in detecting lung cancer.

Several factors are responsible for the failure of pulmonary cytology to have achieved more widespread clinical usage. The continued necessity of examining multiple sputum specimens, the tediousness of the microscopic examination of tenacious mucus, and the paucity of therapeutic successes have conjunctly discouraged the application of pulmonary cytology to large segments of the population. Nevertheless, there is a sizable number of investigators at present whose investigations have established highly reliable morphologic criteria for recognition of pulmonary malignancy and other pathologic chest conditions.[77-87]

The **specimen** may consist of sputum which has been spontaneously expectorated or of bronchial secretion obtained by aspiration or lavage through the bronchoscope. An additional method of collection has been proposed to induce sputum production in asymptomatic individuals by aerosol inhalation of a hypertonic saline solution containing propylene glycol[88] or nebulized SO_2.[89]

Sputum

Thorough instructions to the patient are essential to achieve cooperation and understanding. Failure to clarify instructions often results in obtaining nasal secretion, postnasal drainage, saliva, or an admixture rather than a true sample of lower respiratory secretion. Whenever possible, the patient should be verbally instructed by a member of the cytology laboratory staff; the conventional instruction to "spit in the bottle" does not suffice. Allegedly a fairly large proportion of patients do not raise sputum; however, if the raising of sputum is insisted upon with sufficient firmness, an amount enough for cytologic examination can almost always be collected.[90]

When verbal instruction to each patient is impractical, distribution of written instruc-

INSTRUCTIONS TO PATIENTS FOR SPUTUM CYTOLOGY STUDY*

Sputum is the material that originates from deep in the chest and can be obtained only by deep cough.

Saliva is the watery fluid in the mouth and is **not** desired in this test.

Postnasal drainage is the thick material that may drain down from the nose and collect in the back of the throat, especially during the night in sleep. This material, if present, must be cleared from the throat and **discarded** before coughing to collect sputum for the cytology study.

Instructions:

1. In the morning, before breakfast, clear your throat and **discard** any material that is present.
2. Breathe deeply 8 to 10 times and cough deeply. Collect the sputum obtained in this way in the container provided. Repeat this procedure several times during the first hour after you awake. Even a small amount of material is suitable for this study, provided it has been raised from deep in the chest.

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*Sputum cytology is a special test which requires a fresh morning specimen of sputum to be collected on 5 separate days.

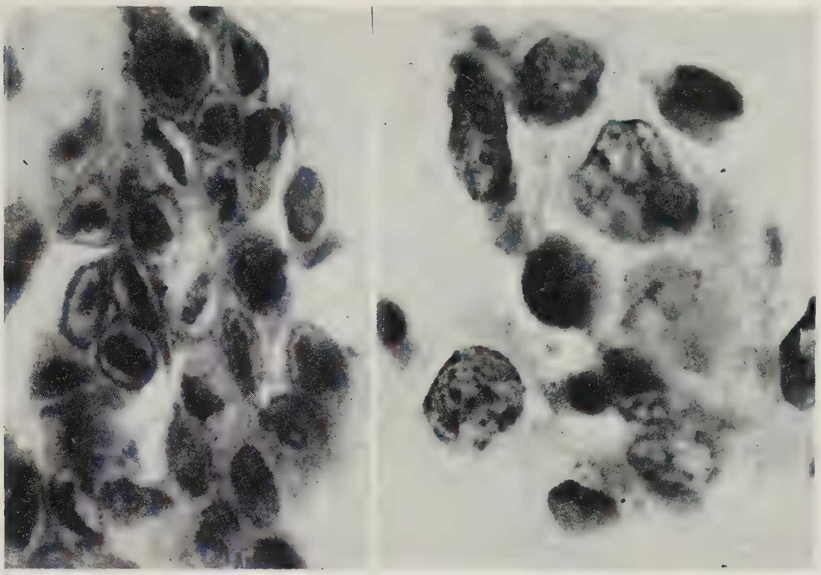

Fig. 592. Cell groups in sputum. Left, dyskeratosis suggesting bronchial squamous metaplasia. Right, hyperkeratinized malignant cells consistent with squamous carcinoma.

tions similar to the accompanying chart is beneficial.

Collection of the sputum specimen into a wide-mouthed glass bottle with a suitable cap or cover is preferred to use of a disposable cardboard carton. The latter is convenient but is less efficient for transferring the specimen to another container for selective smearing or for concentration. The sputum specimen raised upon awakening is best suited for cytologic examination; that raised by spontaneous cough is more likely to yield positive results. Particular effort should be made to obtain the sputum raised during the first hour following bronchoscopic examination.

Smears of sputum should be prepared as soon as possible after its collection; a delay of 4-6 hr. is permitted provided sufficient mucus or saliva is present to delay drying. Papanicolaou[91] recommends collection of sputum in a bottle containing 70% alcohol as a preservative. The resulting hardening of the sputum by alcohol, however, causes difficulty in smear preparation and also in obtaining adhesion of the material to the slide during fixation and staining.

Bronchial aspiration or lavage

The relative merits of cytologic study of secretions obtained by bronchoscopy versus sputum have been widely discussed.[72, 74, 75, 92-96] The reported accuracy of cytologic examination of bronchial aspiration ranges from 30%[75] to 95%.[96] In a series of 500 patients

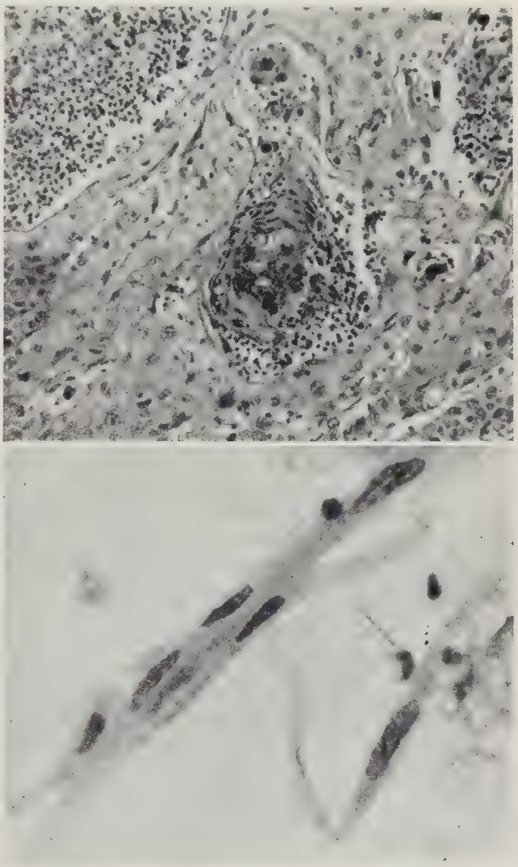

Fig. 593. Above, tissue section, well-differentiated squamous carcinoma of lung with pearl formation. Below, malignant "spindle" cells, assumed to originate from pearl formation.

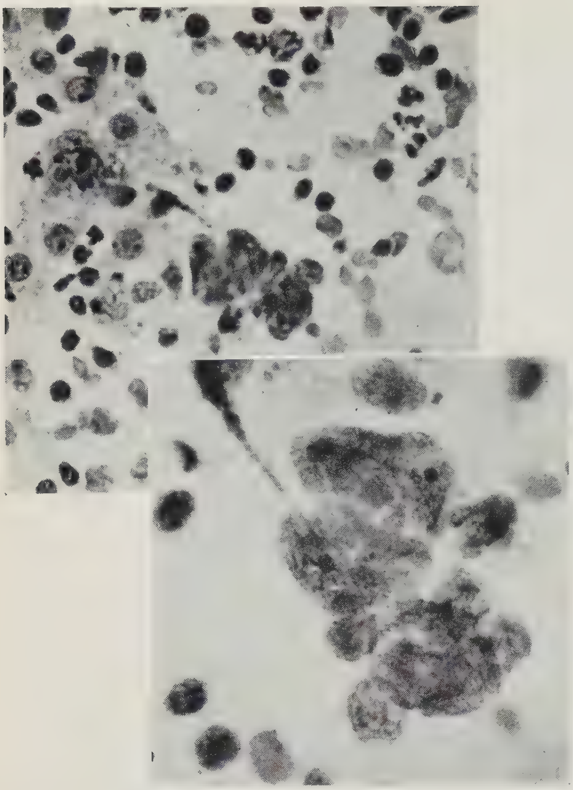

Fig. 594. Dorothy Reed cells in bronchial aspiration in a case of Hodgkin's disease with pulmonary involvement. (Specimen courtesy Dr. Bernard Pierson, Nancy, France.)

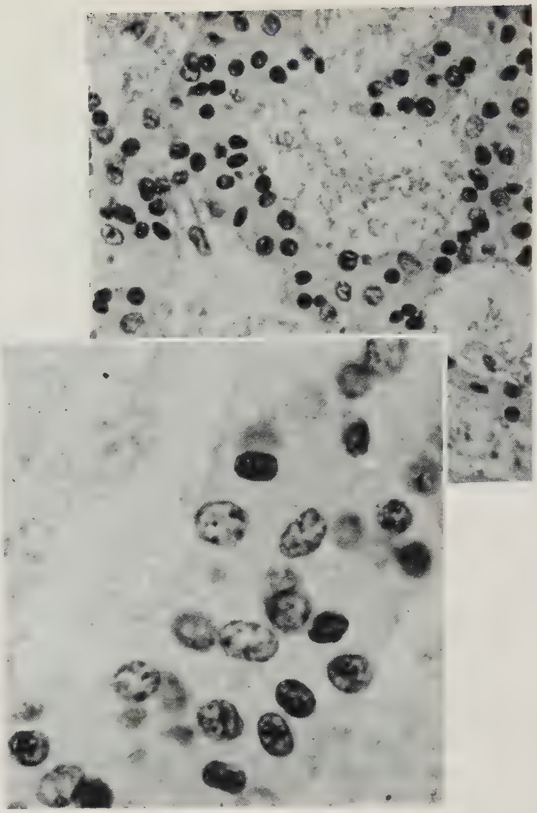

Fig. 595. Sheets of uniform cuboidal cells in bronchial aspiration from a patient with bronchial adenoma. (Specimen courtesy Dr. Bernard Pierson, Nancy, France.)

with bronchial tumors Pierson was able to diagnose 478 (95%) on the basis of bronchial aspiration alone. A repeated bronchial aspiration was required for diagnosis in only 43 of the 478 patients. There were 2 false positive cases among the total series of 4000 cases. Pierson attributes his unusually successful results to the teamwork of bronchoscopist and pathologist for specimen collection. Pierson personally attended bronchoscopy in the majority of cases in his series and insisted upon the following **requirements for specimen collection:** (1) precise location of aspiration with respect to the suspicious area or areas, (2) a slightly traumatizing procedure, and (3) a prolonged aspiration of at least 1 min. from each area examined. It is of further interest to note that Pierson's accuracy dropped by 15% in his examination of specimens referred to him from sources outside his hospital.[97]

Cahan and Farr[98] in 1950 described a method for obtaining secretions or washings from the trachea and main bronchi without bronchoscopy. The procedure can be performed in ambulatory patients.

A small catheter is passed to the trachea and Pontocaine is instilled as the catheter passes to the carina and main bronchi. Two ml. portions of physiologic saline are instilled and reaspirated from various areas and collected for cytologic examination.

The procedure may be useful as a means of obtaining pulmonary secretion from patients who do not raise sputum and whose clinical findings do not warrant bronchoscopy.

METHODS FOR CONDUCTING PULMONARY CYTOLOGIC EXAMINATION

The 3 methods for conducting cytologic examination of sputum and bronchial secretions are (1) direct smear preparations from selected areas of the specimen, (2) cell block preparations, and (3) digestion and concentration of specimens.

Direct smear method

The necessity of examining multiple sputum specimens is a consequence of the many items of chance involved in the collection and preparation of sputum specimens. In spite of giving thorough instructions to patients for collection of sputum, many submit upper respiratory secretions instead of sputum. In addition, no infallible method has been found to identify the types of sputum particles which may harbor malignant cells.

Three smears are prepared from each specimen of sputum or bronchial secretion. An attempt is made to select 3 different types of material from each specimen, with particular interest in searching for bloody strands of mucus, cheesy particles, or tissue fragments. The specimen should be poured into a Petri dish and inspected over a dark background with an adequate overhead light source.

Direct smears are prepared by introducing a pea-sized fleck of carefully selected sputum upon a clean glass slide, using a plain, untipped applicator stick. Excess material clinging to the selected portion of sputum can be severed by "sawing" it against the sharp glass edge with the applicator stick. The isolated portion of sputum can then be streaked across ⅔ of the surface of the slide or, preferably, teased apart and spread to an even thickness with a pair of applicator sticks.

Koss[99] advises use of a small nasal curet or a specially designed shallow spoon* for isolation and transfer of selected sputum particles from specimen to slide. He also advocates use of a "crush" preparation of material between 2 slides, presumably a method preferred for preparation of alcohol-fixed sputum specimens.

Cytologic smears of sputum are often made too thin rather than too thick; either extreme is undesirable.

Immediately after preparation each slide is placed in a fixative solution consisting of equal parts of ether and 95% ethyl alcohol. A paper clip inserted on the identified end of each slide prevents smears from sticking together in the fixative. Place slides in fixative gently and avoid striking container bottom forcefully.

Sputum smears should remain in the fixative for a minimum of 1 hr. before staining.

Accuracy, direct smears: Examination of a single sputum specimen by the direct smearing method is inadequate for lung cancer detection. Farber and Pharr[100] cite their experience of detecting only 45-50% of cases of pulmonary cancer when only 1 sputum sample is examined. A study of 3 separate specimens increases this accuracy to 65%, and a study of 5 separate specimens raises it to 90%. Thus, although cytologic examination of 5 separate sputum samples from each case requires considerable time and effort from trained personnel, study of fewer specimens is of limited clinical value.

Cell block

Some investigators prefer to examine sputum and bronchial secretions by preparing block sections according to the usual histologic technic. According to Hunter and Richardson,[49] the method has the following distinct advantages:

1. Cell dimensions following fixation and embedding in paraffin are the same as those in the more familiar tissue sections.
2. Cell grouping, when present, is not broken up.
3. The small area presented for microscopic examination precludes the necessity for previous screening by a cytotechnician.

More recently, Richardson et al.[101] investigated the results of the simultaneous use of smears and cell blocks in 2613 specimens of various body fluids; approximately ½ the cytologically positive specimens were sputum or bronchial washings (283 of 578 positive specimens). The authors concluded that carefully prepared smears are essentially a better diagnostic medium than cell blocks because of better preservation of cellular detail. They stress, however, that the highest degree of accuracy was achieved by utilizing smears as the principal diagnostic method and supplementing them with cell block preparations of the residual material remaining after smear preparation. With this dual method the authors were able to detect 12% additional cases through cell blocks of residual sputum and gained 19% accuracy by examining cell blocks of residual bronchial washings.

Procedure[101]:

1. Fix specimen in a solution of 13 gm. picric acid in 1000 ml. 70% alcohol for 4 hr. or more.
2. After fixation dehydrate and embed in paraffin.
3. Place 9-12 cut sections on 3-4 slides, stain, and mount.

Bothereau[102] recommends 12-24 hr. fixation in Allen fluid for cell block preparation of body fluids.

Allen fluid[102]:

Picric acid, saturated aqueous solution	75 parts
Formalin, c.p.	15 parts
Glacial acetic acid	10 parts
Urea	1 part

Sputum concentration

Attempts to concentrate the cellular content of sputum and bronchoscopically obtained secretions by mucus digestion have been reported by various investigators[103-109] who have

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*Available from United Surgical Supply Co., Port Chester, N. Y.

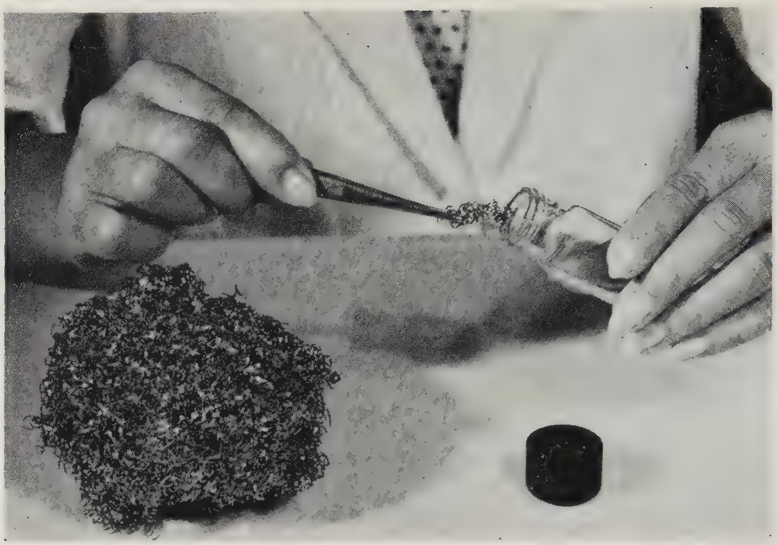

Fig. 596. Strand of metal coil added to sputum before agitation; shreds mucus strands and increases the surface area for effective mucolysis by trypsin solution.

used proteolytic or mucolytic enzymes such as hyaluronidase, lysozyme, papain, trypsin, and chymotrypsin.

The method to be described was developed personally and has been in use for more than 5 yr. A specially selected trypsin product and balanced electrolyte solution as diluent are used with mechanical agitation. Results have consistently been dependable, and there has been no evidence of cellular alterations due to the digestion process either in normal cells or in any type of malignant cells. Cellular grouping is not disjoined, and tinctorial reactions are not altered.

Advantages of tryptic concentration of sputum:

1. Examination of 2 sputum specimens per case is sufficient to obtain reliable cytologic results and is equivalent, if not superior, to examination of 5 or more specimens by the conventional direct smearing method.

2. A sampling from mixed sputum sediment following concentration is representative of the entire specimen, rather than a random selection; thus in each smear the cellular distribution is representative of the cellular population of the entire specimen.

3. Microscopic examination is facilitated by the uniformly thin distribution of smeared cells. Lysis of mucus and the absence of overlying and crowded cells permit improved visualization of cellular and nuclear detail.

4. Risk of laboratory infection is reduced by the method of processing the specimen, and the unsavory task of selecting and smearing sputum is eliminated.

5. Microscopic examination of the homogenized sediment provides a valid index to the relative number and appearance of benign cells and may be of clinical significance in evaluation of nonmalignant chest conditions.

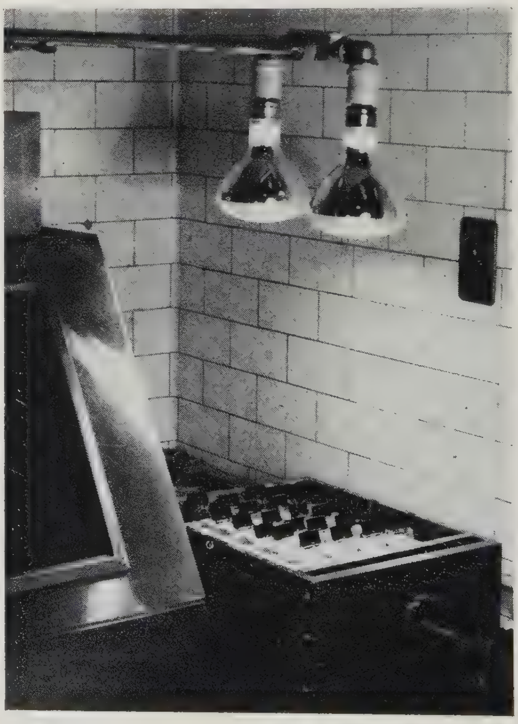

Fig. 597. Successful digestion of mucus by trypsin requires use of forceful agitation and simultaneous heating to 37° C.

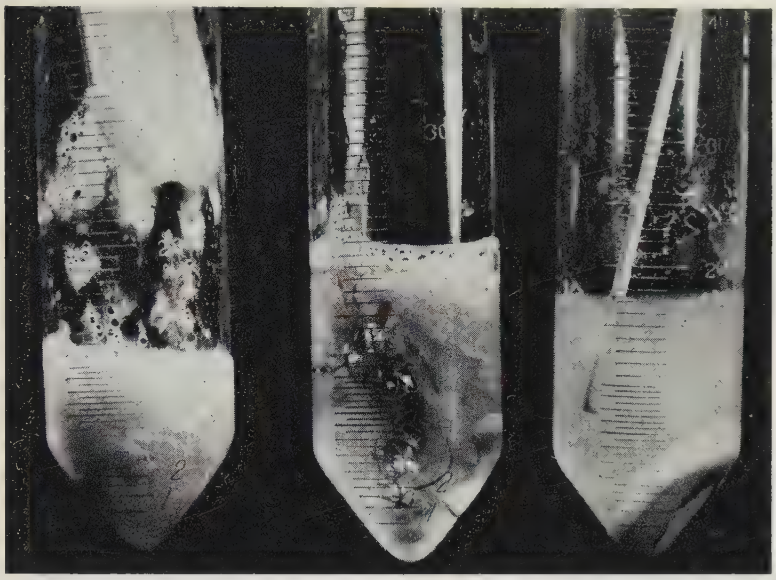

Fig. 598. Tryptic concentration of sputum. Left, tenacious sputum sample before treatment. Center, same specimen after addition of trypsin solution and metal coil. Right, same specimen following agitation and centrifugation. Note separation of sediment into distinct layers; the uppermost "buffy" layer is almost entirely bacteria.

The examiner is able to comment meaningfully on purulence, eosinophilia, and polymorphonuclear ratio and to judge semiquantitatively the content of macrophages, columnar cells, erythrocytes, and other elements in sputum samples.

6. Concomitant bacteriologic and mycologic laboratory examinations can be performed on the sediment processed for cytologic study with a resulting sensitivity that is equivalent, if not superior, to that of standard methods of study.

Concentration of the specimen requires approximately 60 min. Although this is considerably longer than the few moments needed to prepare direct smears, no attention from the worker is required during 45 of the 60 min. In addition, multiple specimens can be processed at the same time.

Reagents:

1. Crystalline trypsin.*
2. Isolyte-E, without dextrose.†
3. Celloidin-ether-alcohol fixative.
   (a) Add 1.5 ml. thick celloidin to each liter of equal parts of ether and 95% ethyl alcohol.
4. Papanicolaou staining solutions.‡ The Pharr, Wood, and Traut modified counterstains and modified staining method are particularly well suited for staining concentrated sputum sediment smears (pp. 1765 and 1766).

Equipment:

1. Box type shaking machine.
   260-270 strokes/min., 1½-2½ stroke amplitude. Rotator types not sufficiently forceful for use.
2. Pair of 250-watt infrared heat lamps.
   Mount on adjustable stand over shaking machine.
3. Stainless metal sponge, ribbon gauge.
4. 50 ml. round-bottomed centrifuge tubes with screw cap.*
5. Pasteur capillary pipets, 9 in. length.†

Procedure: The technic that follows applies to processing specimens of 10 ml. volume or less. Larger samples are divided into approximately 10 ml. portions and processed separately.

1. Measure 10 ml. Isolyte-E for each specimen; e.g., for processing 8 specimens, measure 80 ml.
2. Weigh 10 mg. crystalline trypsin for each specimen; e.g., for 8 specimens, weigh 80 mg.
3. Mix trypsin and Isolyte-E immediately before procedure is begun. **Caution:** There should be no lengthy delay after this point.
4. Add 10 ml. enzyme-electrolyte solution to each specimen, mix with a tongue blade, and transfer to a centrifuge tube, draining the entire liquid portion.
5. Add a 1 in. strand of metal ribbon cut from a scrubbing sponge and cap the tubes.

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*Takamine Laboratory, Clifton, N. J. Variable results may be found with other brands of crystalline trypsin.

†Solution available as Plasma-Lyte from Baxter Laboratories, Inc., Morton Grove, Ill.

‡Available from Gradwohl Laboratory Supply Co., St. Louis, Mo.

*Corning Glass Works, Corning, N. Y.; Cat. No. 8422.

†Scientific Products; Cat. No. 67771.

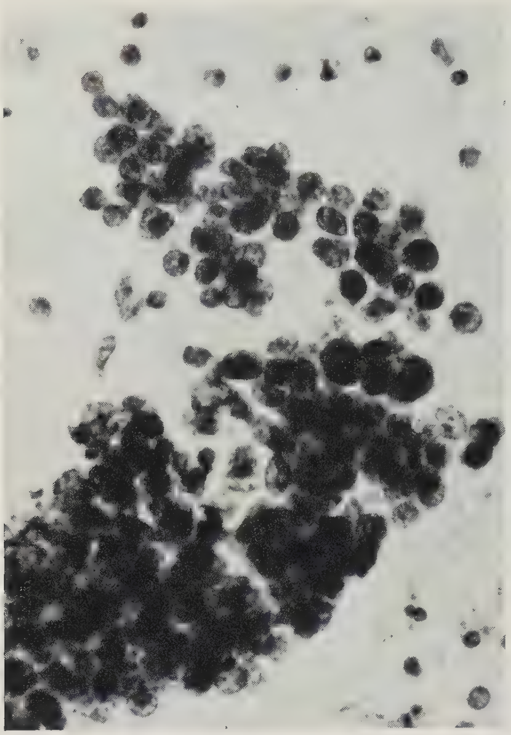

Fig. 599. "Oat cells" in tryptic concentrated sputum smear.

6. Place the tubes in a shaking machine at a 45° angle and agitate for 30 min. Overhead heat lamps are placed at a fixed distance to maintain a temperature of 35°-40° C. within the tubes during digestion.
7. Following digestion, remove the metal coil from each tube and discard. Without delay recap the tubes, balance, and centrifuge for 15 min. at 2500 r.p.m. Allow the tubes to come to rest without braking.
8. Pour off the supernatant fluid with a single rapid inversion and allow the tubes to drain for a full minute.
9. Mix the sediment **thoroughly** and prepare cytologic smears by the pull-apart method, previously described (p. 1760).

Concomitant laboratory uses of tryptic concentrated sputum: The use of trypsin as a digestant of sputum for isolation of acid-fast bacilli was described by Haynes[110] in 1942. The previously described tryptic digestion method was designed to preserve cellular detail but has also proved to be an efficient means for concentrating bacteriologic and mycologic components of sputum.

A top buffy layer is present in the sediment of most tryptic concentrated sputum specimens, and this has been found to contain a superconcentration of bacteria and light, amorphous debris. Material collected by loop or pipet from the buffy layer prior to mixing of the sediment for cytologic smear preparation is an excellent source of bacteria for bacteriologic examination by smear or by culture. Mycologic smears and cultures can be obtained from the mixed sediment.

Following is a résumé of laboratory examinations of tryptic concentrated sputum:

1. Acid-fast stain—1 slide from buffy layer.
2. Gram stain—1 slide from buffy layer.
3. Papanicolaou stain—2 or more slides from mixed sediment.
4. Fungus stain—1 slide from mixed sediment.
5. Tubercle bacilli culture—inoculate from buffy layer or from treated mixed sediment.
6. Pyogenic culture—inoculate from buffy layer.
7. Fungus culture—inoculate from mixed sediment.

GASTROINTESTINAL CYTOLOGY

Many malignant lesions of the gastrointestinal tract occur in locations which are accessible to cytologic examination by use of specialized technics. Although the methods employed for collection of gastrointestinal cytologic specimens are relatively simple, safe, and applicable to ambulatory patients, they are nonetheless time-consuming procedures and often demand patient selection on the basis of clinical symptoms or findings.

Esophagus

Rubin[111, 112] states that cytologic examination of esophageal secretion appears to be the most accurate of all gastrointestinal cytologic procedures. He and associates were able to detect 46 of 47 (97.9%) cases of malignant lesions of the esophagus by a lavage technic. In 3 additional reports by Gephart et al.,[113] Raskin et al.,[114] and Klayman[115] accuracy in detecting malignant lesions of the esophagus ranged from 90.5 to 95%. Imbriglia and Lopusniak[116] have reported cytologic detection of carcinoma in situ of the esophagus in 1 case, and Koss[117] reports an additional case detected cytologically as "approaching in situ carcinoma."

Rubin[111] has emphasized that the best results can be expected when the same physician is responsible for specimen collection and microscopic interpretation. He also indicated the need for familiarity with special clinical situations, such as vitamin B_{12} or folic acid deficiency, prior radiation, inflammatory disease, and others, to avoid false positive diagnoses of malignancy.

Lavage[111]

The patient should fast for 12 hr. before the cytologic examination. A 48 hr. delay is required after barium ingestion. In the case of a severely obstructed esophagus, a clear liquid diet may be necessary for several days preliminary to lavage.

If no obstruction is encountered, a Levin tube is passed into the stomach with the patient in the seated position.

The gastric contents are then aspirated and discarded unless tissue fragments are present, in which case they are isolated and smeared.

The patient is cautioned to expectorate all saliva and mucus of respiratory or nasal origin during the procedure.

The patient then drinks 100 ml. Ringer solution, which is recovered from the stomach.

With the patient in the supine position, the tube is withdrawn 5 cm. at a time.

At each level 50 ml. Ringer solution is vigorously instilled and reaspirated. If a questionable area has been previously localized by x-ray examination, special effort is made to lavage this area thoroughly.

Immediate centrifugation of the lavage specimen is essential, and smear preparation and fixation must follow without delay.

Abrasive cell collection

Various abrasive methods for esophageal cell collection during esophagoscopy have been described[118-121] and include use of a gauze sponge or specially designed abrasive instruments. Conflicting opinion exists regarding the reliability and advisability of employing such methods in cases of suspected esophageal cancer.[111, 113]

Stomach

The stomach is easily accessible for collection of cellular material. The reliability and accuracy of gastric cytology to detect up to 95% of cases of gastric cancer have been proved in a number of reports in which large series of patients have been examined.[122-128]

The methods described for obtaining gastric material for cytologic examination have varied widely from simple aspiration of fasting gastric contents to lavage by various solutions and use of abrasive instruments. Studies comparing the accuracy of various methods indicate that equally sensitive results are obtainable by each method, provided meticulous care is taken in performing the procedure.[129, 130] Rinaldo et al.[131] indicate that gastric cytology is especially useful for detection of cancer in the antrum, on the greater curvature, and in the fundus, and they note that it is in these areas that the radiologist most frequently fails.

Saline lavage[130]

Part I:

1. Have the patient fast overnight. In the morning, with the patient sitting upright on a bed or cot pass an iced No. 18 Levin tube orally 55 cm. into the stomach.
2. Aspirate and discard the gastric residuum.
3. Quickly and **forcefully** inject and aspirate 100 ml. isotonic saline. Then reinject and reaspirate, collecting in centrifuge tubes that are immersed in an ice-water bath.
4. Allow the tube to remain in place and inject 100 ml. fresh saline, repeating the procedure as described.
5. Repeat until 300-400 ml. fluid have been used.

Part II:

1. In the second part, introduce 500 ml. isotonic saline into the stomach.
2. Have the patient lie in each of the following positions for 2 min. each: on the right side, back, abdomen, and finally on the left side. Briskly massage the upper abdomen while the patient is supine.
3. Withdraw fluid when the patient has completed a 360° rotation. Approximately 400 ml. can be recovered.
4. Centrifuge the aspirate immediately and prepare and fix the smears without delay.

Comment: Ringer solution or a buffered solution may be substituted for isotonic saline. All authors have stressed the necessity of performing a vigorous and aggressive lavage and have emphasized the necessity of centrifuging the specimen and preparing smears as rapidly as possible after collection. Use of a bedside, portable centrifuge has been recommended.

Enzymatic gastric lavage

Two mucolytic enzyme solutions have been described for use as lavage solution in gastric cell collection. The purpose of each is to dissolve the mucous barrier which coats the luminal surface of the stomach and thus to permit recovery of cells trapped in the mucus and, more importantly, to permit shedding of cells directly from the surface of a gastric lesion.

Papain, a proteolytic enzyme derived from the fruit of the papaw, was used with a buffered solution for gastric lavage by Rosenthal and Traut,[122] and their method was reported in 1951. In 1955 Rubin and Benditt[132] introduced a similar lavage procedure which utilized another enzyme, α-chymotrypsin.

No superiority in results has been conclusively indicated for either method. The preparation of α-chymotrypsin lavage solution has been greatly simplified by use of a commercially prepared capsule containing a measured quantity of enzyme.*

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*A capsule containing 7 mg. crystalline α-chymotrypsin available as Cyto-Lav from Armour & Co., Kankakee, Ill.

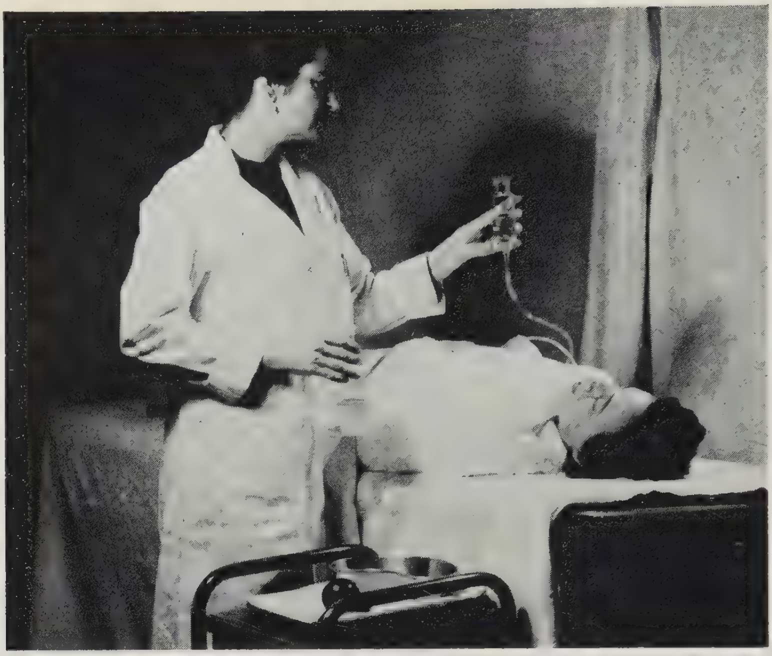

Fig. 600. Five hundred milliliters papain solution are instilled into stomach slowly.

Papain lavage—Rosenthal-Traut[122]

Reagents:

1. Papain buffer solution.

Dibasic sodium acid phosphate	350 gm.
Hydrochloric acid, concentrated	33 ml.
Distilled water	5 gal.

   (a) The pH is 7.3.
   (b) The buffer will keep indefinitely.

2. Papain gastric lavage solution.
   (a) For each lavage place 500 ml. papain buffer solution in a mechanical stirrer such as a Waring blendor and add 3 rounded teaspoonsful of powdered papain.
   (b) Mix 3-5 min. During the last 1 min. add 1 heaping spoonful of powdered activated charcoal.
   (c) Filter. The first few drops will contain charcoal; return these to the funnel. The remaining filtrate should be sparkling clear and amber.
   (d) Papain lavage solution can be kept 24-48 hr. before use if refrigerated.

Method: Preparation of the patient and intubation conform to previous instructions for conducting saline lavage. At least 2 wk. should elapse after gastric hemorrhage before the test is made.

1. Aspirate and discard fasting gastric contents.
2. Dissolve ¾ teaspoon cysteine hydrochloride (activator) in 500 ml. papain lavage solution immediately before use.
3. Instill 500 ml. activated papain lavage solution into the stomach by tube.
4. Request the patient to lie down and to rotate position frequently during the next 10 min. Gentle massage may be applied in the area of the stomach.
5. After 10 min. withdraw the lavage solution by gravity flow and centrifuge as soon as possible.
6. Underlay 2-3 ml. serum or plasma in the specimen before centrifugation to ensure adhesion of smeared sediment.
7. Make 4-6 smears by the pull-apart method.

Alpha-chymotrypsin gastric lavage

Reagent:

1. Sodium acetate buffer, pH 5.6.

Sodium acetate, crystalline	13.6 gm.
Glacial acetic acid	0.6 ml.
Distilled water	1000 ml.

   (a) Prepare the buffer in small quantity and refrigerate.

Method—Rubin et al.[112, 132]: Patient preparation and intubation are the same as previously described for saline lavage.

1. Prepare the α-chymotrypsin lavage solution immediately before use by adding the contents of a Cyto-Lav capsule* containing 7 mg. crystalline α-chymotrypsin to 500 ml. sodium acetate buffer.
2. Instill into the stomach the entire 500 ml. or as much as will be tolerated by the patient. Ask the patient to rotate himself through 360° during the next 10 min.

The solution should not remain in the stomach longer than 10 min. and should be withdrawn and centrifuged as quickly as possible. No more than 10 min. should elapse between withdrawal of fluid and fixation of smears.

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*A capsule containing 7 mg. crystalline α-chymotrypsin is available as Cyto-Lav from Armour & Co., Kankakee, Ill.

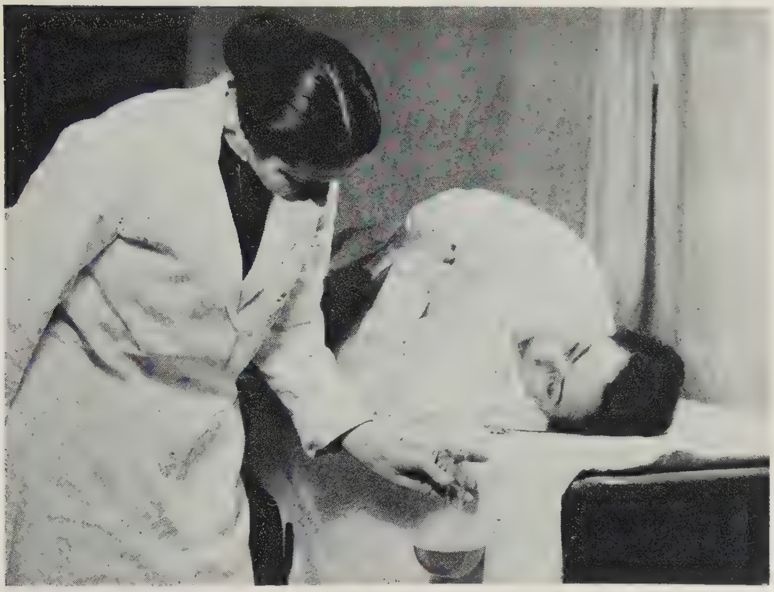

Fig. 601. At the end of 10 min. solution is recovered by gravity flow.

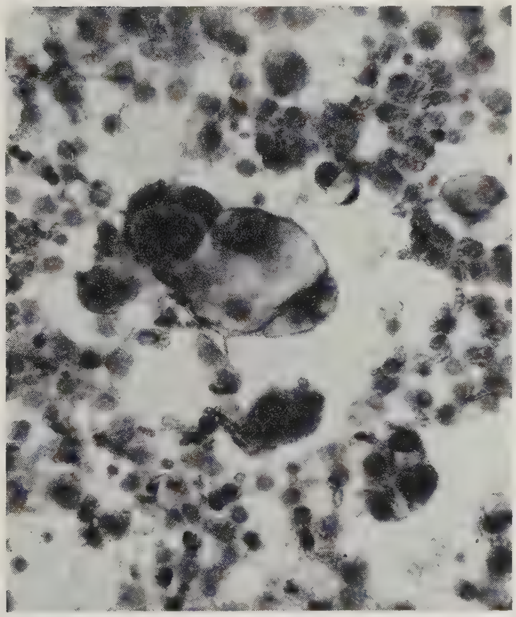

Fig. 602. Several groups of malignant cells in papain gastric lavage smear. Diagnosis was adenocarcinoma of stomach.

Abrasive methods for gastric cell collection

Sponge biopsy: A number of methods have been described for gastric cell collection, which depend upon mechanical irritation and abrasion of the gastric mucosa. The first method was described by Gladstone[133] in 1949. The method utilizes a Levin tube, along the lower portion of which 12-15 pieces of synthetic cellulose sponge are wound.

1. Pass the tube into the stomach and, with the patient on the right side, massage the abdomen to bring the sponges into contact with the gastric mucosa.
2. After withdrawal, fix the sponges in 10% formalin, embed in paraffin, and section according to standard histologic technics.

Gladstone has described similar applications of his sponge biopsy method for cell collection from cervix, rectum, bronchus, oral cavity, and skin.

Gastric balloon: The gastric balloon method of gastric cell collection was described by Panico et al.[134] in 1950. The instrument is a specially designed, inflatable gastric balloon covered externally with a wide-meshed silk net.*

1. Pass the balloon orally into the stomach, and then inflate and draw it to the cardia. Peristaltic action returns the balloon to the pylorus in 5-10 min.
2. Repeat the procedure each 10 min. for 30 min.; then deflate and withdraw the balloon.

Cellular material is obtained by vigorous rinse of the balloon and net in Ringer solution.

Centrifugation of rinsing fluid and smear preparation must follow without delay.

Cooper and Papanicolaou[135] report an overall diagnostic accuracy of 97.1% with the

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*The entire apparatus can be purchased from Dr. Arthur O. Dahlberg, Walbrook Road, Scarsdale, N. Y. Full directions are given with the instrument.

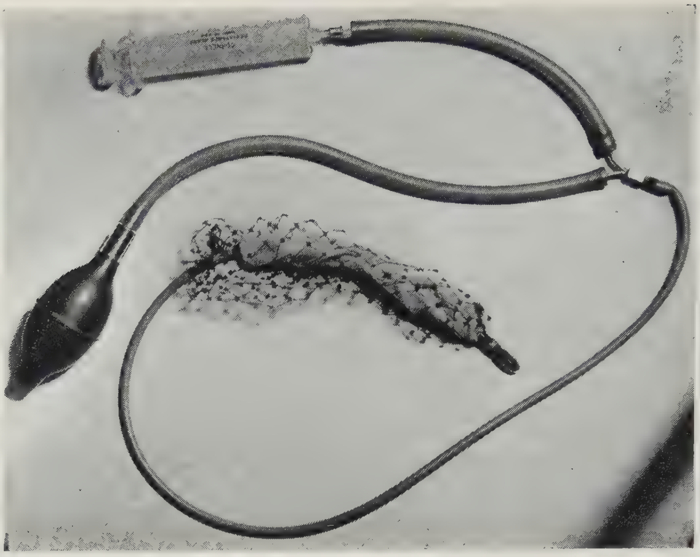

Fig. 603. Inflatable gastric balloon apparatus of William A. Cooper, Frederick G. Panico, and George N. Papanicolaou.

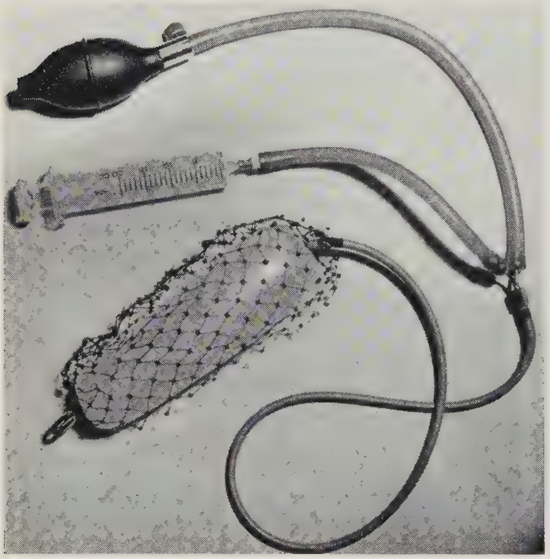

Fig. 604. Inflatable gastric balloon apparatus, showing the balloon partially inflated.

abrasive balloon technic. Other investigators[128, 136, 137] have found the technic to be prohibitively time-consuming and not tolerated well by patients. Koss[137] states that the abrasive balloon method is preferable to lavage only in cases of advanced gastric tumors with necrotic surfaces.

Abrasive brushes: Two abrasive brush instruments for gastric cell collection have been described. The first was reported by Ayre and Oren[138] in 1953. This is a retractable, biwinged brush which is spread open after passage to the stomach.

1. Rotate the brush as the tube is withdrawn from the pylorus to the cardia. Close and retract the brush and then withdraw the tube.
2. Prepare cytologic smears from material obtained by squeezing the brush from its base to tip between 2 slides.

Willner et al.[139] state that an unsatisfactory number of false positive diagnoses of malignancy are associated with gastric cytologic examinations obtained by the Ayre brush method and attribute the difficulty to misinterpretation of benign sheets of gastric mucosal cells. Nieburgs[140] has described a modified Ayre brush composed of retractable loops of nylon fibers.

Duodenum and pancreatic and biliary systems

Bowden and Papanicolaou[141] examined secretions obtained from the duct of Wirsung at the time of performing pancreatoduodenectomy in a small series of patients. They reported that they could usually identify exfoliated malignant cells in the fluid in cases of primary infiltrating carcinoma of the pancreas and in cases of carcinoma of the common bile duct or duodenum secondarily invading pancreatic parenchyma.

Cytologic examination of secretions obtained by duodenal drainage has remained of limited clinical value, however, because of the specialized skill that is required for specimen collection. Duodenal secretion is often scanty and its microscopic interpretation is difficult.[123, 142]

In a series of 356 duodenal drainages, Raskin et al.[143] were able to detect 33 of 55 cases (60%) proved to have carcinoma of the pancreas, bile ducts, gallbladder, or primary or secondary duodenal carcinoma. A similar accuracy has been reported by other investigators.[112, 144-146]

Colon and rectum

Cytologic examination of colonic washings is valuable for detection of malignant lesions beyond the reach of the sigmoidoscope. Rigorous preparation by cathartic and enemas is essential. An assumption that diagnostic malignant cells are removed during cleansing enemas is not substantiated by the reports of highly successful cytologic studies of colonic and rectal washings.[123, 147-150] Raskin et al.[123] obtained positive cytologic examinations in 36 of 38 cases (95%) with proved colonic malignancy. In 10 of the 36 patients with positive cytologic findings the initial barium enema examination failed to disclose evidence of malignancy.

Procedure—Koss[151]

1. Give the patient 2 oz. mineral oil or castor oil by mouth 12 hr. prior to examination.
2. On the morning of the procedure, administer cleansing enemas until returns are clear. Collection of cytologic material may be delayed 1-2 hr.
3. Instill 500-1000 ml. warm Ringer solution with the patient in the left decubitus position. Massage of the colon and rotation of the patient are helpful in obtaining better cytologic material.
4. Collect the fluid after 3-5 min., centrifuge immediately, and prepare and fix smears without delay.

SEROUS FLUIDS

The accumulation of fluid within the pleural, peritoneal, or pericardial cavities is indicative of a pathologic process. With the rare exception of mesothelioma, the presence of malignant cells in serous fluid indicates metastatic involvement. The appearance of serous effusion is often the first indication of a primary malignancy elsewhere and, as a result, considerable interest has been directed to its examination by the cytologic method.

Monographs by Whiman[152] and Spriggs[153] and a number of descriptive articles have been published which give detailed accounts of cellular morphology in both benign and malignant effusions.[154-161]

Serous fluids should be collected into an anticoagulant solution and rotated throughout collection. Addition of an anticoagulant agent after the specimen is collected will usually not prevent clotting of sediment following centrifugation. Any standard anticoagulant used for blood collection may be used and in the amounts required for the same volume of blood. Sodium citrate is economical and satisfactory; approximately 10 ml. 3.8% sodium citrate are used for each 100 ml. fluid collected. Ethylenediamine tetraacetate (EDTA) is highly recommended for use as an anticoagulant in serous fluids because of its excellence in preserving cellular morphology and in preventing the formation of fibrin. The dipotassium salt is preferred to the disodium salt for cytologic usage.

An aliquot of 100-200 ml. is the minimum requirement for cytologic examination. Addition of an adherent agent is not required. Following centrifugation, particular effort should be made to collect material from the top buffy layer for smearing; this layer is especially conspicuous in specimens of bloody effusion and is a rich source of malignant cells. The pull-apart method of smear preparation (p. 1760) gives excellent results.

When absolutely necessary, a serous fluid specimen may be refrigerated overnight before processing; however, artifactual changes, such as vacuolization and cytolysis, may provoke uncertainty in the microscopic examination. Collection of the specimen directly into an equal volume of 50% alcohol or 10% aqueous formalin has also been advocated.

The cell block technic is often employed as

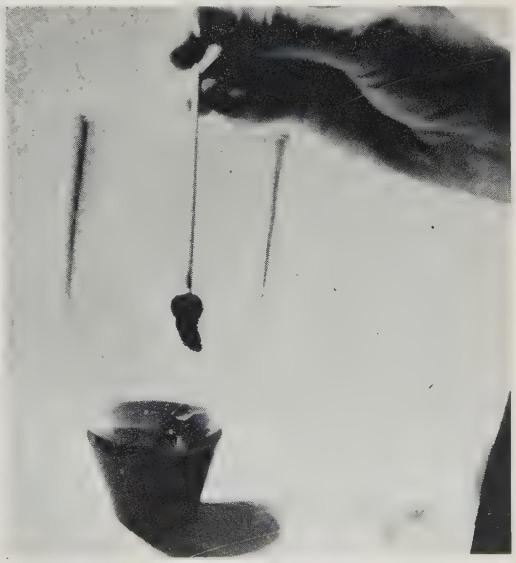

Fig. 605. Clotted serous fluid sediment caused by failure to collect specimen into anticoagulant.

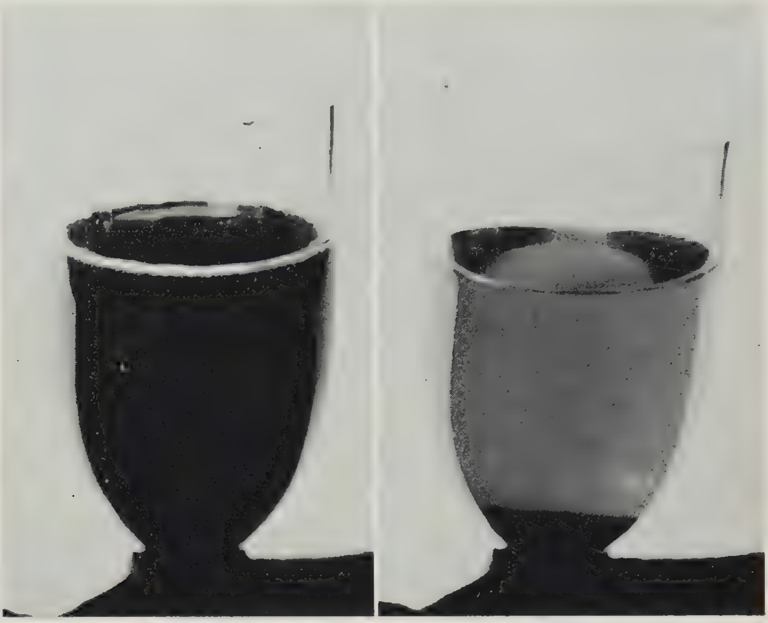

Fig. 606. Bloody pleural fluid before (left) and after (right) centrifugation. Note top "buffy" layer which is a rich source of malignant cells.

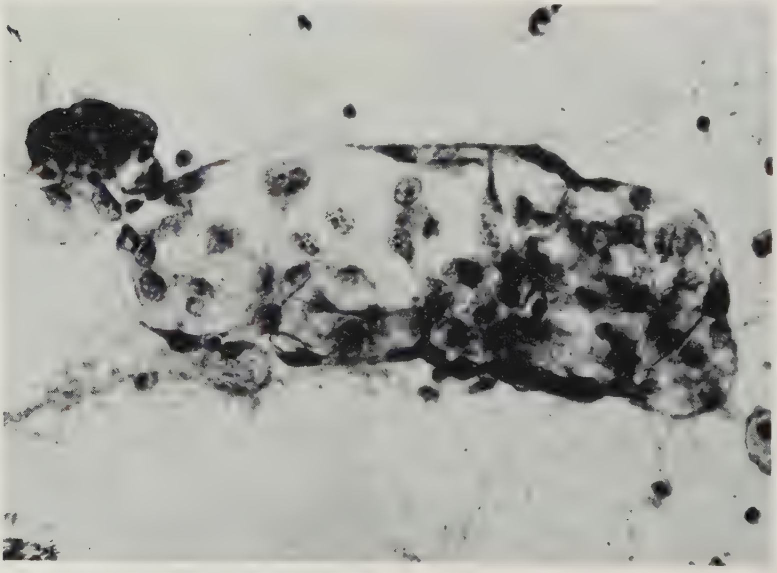

Fig. 607. Large, glandular group of malignant cells in ascitic fluid. The site of the primary was not determined.

an additional method of examining serous fluid sediment.[160, 162]

Rapid examination of serous fluid sediment can be performed through use of supravital staining. Foot and Holmquist[163] described their results with supravital staining by an alcoholic solution of neutral red and Janus green on a warm stage. A 5% solution of toluidine blue or brilliant cresyl blue in physiologic saline may also be used.

1. Place a drop of serous fluid sediment on a clean glass slide and mix with an equal volume of freshly filtered supravital staining solution.
2. Cover the wet smear with a cover glass and examine after 2-5 min.

The method is purely one of interest to the cytologist; the final diagnosis should await examination of the permanent Papanicolaou-stained smears.

Ceelen[164] and Foot,[165] in separate studies, investigated the use of the periodic acid-Schiff (PAS) staining reagent for differentiating benign mesothelial abnormalities from malignancy. Foot's application of Mowry alcian blue periodic acid-Schiff stain can be applied to smears previously stained by the Papanicolaou method.

URINARY SEDIMENT

Voided urine from males is suitable for cytologic examination; a catheterized specimen should be obtained from females. The first morning specimen is the most cellular, but cells are greatly distorted because of prolonged retention in the bladder. It is perhaps advisable to collect both an early morning specimen and one later in the day. In either case the specimen must be centrifuged and smears of the sediment prepared and fixed as rapidly as possible after collection.

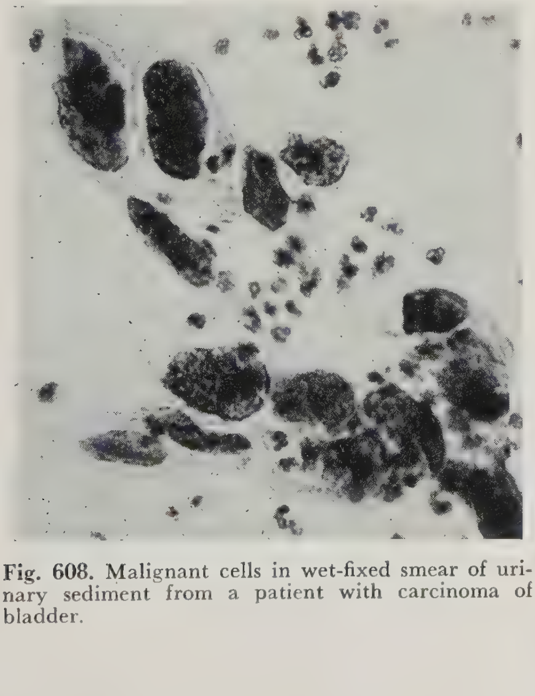

Fig. 608. Malignant cells in wet-fixed smear of urinary sediment from a patient with carcinoma of bladder.

A significantly large proportion of urinary cells is detached during fixation and staining resulting in a large number of unsatisfactory smears and false negative diagnoses. The reported incidence of unsatisfactory urine smear examinations has ranged from 20 to 30%.[166-168] Feeney et al.[169] reported in 1958 that insufficient cytologic evidence of malignancy was observed in 58% of cases with known urinary tract cancers.

A variety of methods has been proposed to improve adhesion. Partial to complete drying in the air has been suggested, followed by rehydration with sodium lauryl sulfate (see rehydration method, p. 1763). Solomon et al.[170] compared results obtained by immediate fixation in ether-alcohol, air drying with rehydration, and air drying without rehydration. They found, as was expected, that air-dried smears showed appreciably better adhesion but poorer cellular preservation than wet fixed ones. They found no appreciable difference between rehydrated smears and those that were not rehydrated. They advocate simple air drying before staining and report that results are suitable for reliable evaluation. Rofe[171] also advises use of the air drying method but recommends addition of a small amount of 8% glycerin solution at pH 7 to protect the cells from distortion due to drying.

The Millipore filter* has been applied to collection of cells suspended in urine. The method was first described by Seal in 1956[172] for collection of cells in serous fluids. A specially designed filter membrane of great porosity is used. The pore size is exceptionally uniform and is available in diameters ranging from 10 to 5μ. Fluid passes through the membrane rapidly; formed elements larger than the pore diameter are retained on the membrane and adhere to it. The entire membrane (4.7 cm. in diameter) is then stained by hematoxylin and eosin or by the Papanicolaou method. The membrane is rendered transparent in xylol before mounting. A detailed account of the Millipore filtration method for processing urine and special instructions for staining the membrane are contained in reports by Solomon et al.,[173] Merritt et al.[174] and Harpst et al.[175]

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*Millipore Filter Corp., Watertown, Mass.

BREAST SECRETION

Spontaneous nipple discharge is collected with a cotton-tipped applicator stick and is quickly spread with a rolling motion onto a clean glass slide. The smear must be placed in the fixative without delay. If the secretion is scant, restrict the smear to a small area of the slide to avoid drying before fixation. If secretion is obtained from both breasts, identify smears as left and right.

In the case of localized lesions of the breast which produce discharge on pressure, the secretion can be collected directly on the slide after expressing material from this area. The pull-apart method of smear preparation (see methods of smear preparation, p. 1760) is well suited for smearing breast secretion.

Detailed accounts of cytologic findings in benign and malignant breast secretions have been reported.[176-179]

PROSTATIC SECRETION

Secretion expressed from the prostate by massage is collected directly upon a clean glass slide. It is advisable to have the patient hold the slide to assure recovery of the secretion. Cytologic examination of urine voided after prostatic massage is advisable, particularly if the massage was unproductive.

Highly accurate results have been reported. In a series of 100 patients with proved prostatic cancer, Peters and Frank[180] reported positive or suspicious cytologic evidence of malignancy in 95. Riaboff,[181] on the other hand, states that in his experience cytologic examination of prostatic secretion from asymptomatic individuals is of quite limited value. The necessity for thorough familiarity with cellular appearances in benign and malignant conditions has been emphasized.[182-184]

ORAL SMEARS

The cytologic method has been applied successfully to the detection of cancer of the oral cavity. The method is particularly valuable when used routinely to investigate all lesions of the oral or oropharyngeal mucosa, regardless of how insignificant the lesion may appear.

Cell collection is obtained by moderately forceful scraping of the lesion with a tongue blade or similar dull instrument. When present, superficial necrotic material and pus should first be removed. The material that can be obtained is often scant; therefore care must be taken to smear the secretion quickly over a small area of the slide. Fixation must follow without delay.

Sandler[185] in 1961 reported 91% agreement in cancer diagnosis between cytology and biopsy in a cytologic study of 452 abnormalities of oral mucosa. In 7 cases multiple biopsies were required to confirm the cytologic diagnosis of cancer; 1 case required 5 biopsies. Montgomery and von Haam[186] reported a 93% accuracy in diagnosis of oral malignancy by smear examinations; other investigators[187-189] have reported similar success.

Oral smears for determination of sex genotype

In 1949 Barr and Bertram[190] noted that the nuclei of neurons of female cats possessed single, small, densely staining, crescent-shaped masses of heterochromatin attached to the nuclear membranes. These masses were absent in the male. Subsequent studies disclosed that similar dimorphism distinguished the nuclei of human male and female subjects. Almost all tissues of the body have been examined for sex chromatin determination with consistent confirmation of Barr and Bertram's observation. Cell collection from the oral mucosa is convenient and suitable for demonstration of the sex chromatin.

The determination of genetic sex has been applied to establish the true sex of hermaphrodites and has also been of interest in studies of teratomas. A nucleus possessing the sex chromatin may be described as chromatin-positive, whereas one without is said to be chromatin-negative.

Normal values are as follows[191]:

Normal males: 0-4% of nuclei are chromatin-positive.

Normal females: 20-80% of nuclei are chromatin-positive.

Procedure:

1. Use a tongue blade, glass slide, or metal spatula for cell collection.
2. Draw the instrument firmly over the oral mucosa and smear evenly on a clean slide the milky fluid that collects.
3. Confine the smear to a small area of the slide, if scanty material is obtained, and place in a fixative immediately.
4. Collect at least 4 smears, repeatedly scraping the same area of oral mucosa.
5. Oral smears for sex genotype determination may be stained by the Papanicolaou method, by hematoxylin and eosin, or by the Guard stain, the latter specifically designed for demonstrating the sex chromatin.

Guard stain for sex chromatin[192]:

Staining solutions:

I. Biebrich scarlet stain:

Biebrich scarlet (Harleco)	1 gm.
Phosphotungstic acid, c.p.	0.3 gm.
Glacial acetic acid	5 ml.
Ethyl alcohol, 50%	100 ml.

II. Fast-green FCF stain:

Fast-green FCF (Harleco)	0.5 gm.
Phosphomolybdic acid, c.p.	0.3 gm.
Phosphotungstic acid, c.p.	0.3 gm.
Glacial acetic acid	5 ml.
Ethyl alcohol, 50%	100 ml.

Guard procedure:

1. Following ether-alcohol fixation, transfer slides to 70% alcohol for 2 min.
2. Stain in Biebrich scarlet staining solution for 2 min.
3. Rinse in 50% alcohol.
4. Differentiate in fast-green FCF solution for approximately 4½ hr. Check under a microscope at hourly intervals. Differentiation is complete when all the cells and nuclei are green, with the exception of pyknotic nuclei which will remain bright red. The sex chromatin stains pink to red.
5. Rinse in 50% alcohol for 5 min.
6. Pass through 70%, 95%, and absolute alcohol, 2 min. each.
7. Clear in 3 changes of xylol, 2 min. each, and mount.

CEREBROSPINAL FLUID

The volume of cerebrospinal fluid that can be withdrawn for cytologic examination is small and often contains few cells. McCormack et al.[193, 194] described a wet-film method for cytologic examination of cerebrospinal fluid sediment, which permits microscopic examination of scanty sediment by addition of a drop of toluidine blue staining solution. The method follows.

Wet-film examination of cerebrospinal fluid—McCormack et al.[193, 194]

Reagents:

1. Stock toluidine blue solution.

Toluidine blue	1 gm.
Ethyl alcohol, 95%	20 ml.
Distilled water	80 ml.

   (a) Grind the stain in a mortar with the alcohol and add the water slowly.
   (b) Store in a glass-stoppered bottle.

2. Toluidine blue staining fluid.

Toluidine blue stock solution	10 ml.
Equine serum	10 ml.

   (a) Centrifuge the mixture. The supernatant fluid constitutes the staining solution.
   (b) Add a few crystals of thymol and store in a refrigerator. Stable for several months.

Procedure:

1. Centrifuge freshly collected cerebrospinal fluid for 10 min. at 2000 r.p.m.
2. Decant the supernatant fluid and add 1 drop of toluidine blue staining fluid to the sediment and mix.
3. Transfer a drop of the stained sediment to a glass slide and cover with a cover glass.

NOTE: After wet-film examination, place the cover slips in acetic acid-alcohol fixative (97 ml. 95% ethyl alcohol, 3 ml. glacial acetic acid). Then stain the slide and cover glass by the Papanicolaou method and remount.

BONE MARROW

Bone marrow is a frequent site for metastasis of primary tumors elsewhere, especially carcinoma of the breast, lung, thyroid, kidney, prostate, gastrointestinal tract, and malignant melanoma. Hyman[195] has reported that only 57% of patients whose bone marrow contained cytologic evidence of malignancy had diagnostic lesions detected by skeletal x-ray survey. On the other hand, he reported that in a control group of 100 patients in whom marrow aspirates were negative for tumor cells, 30% had positive skeletal surveys for tumor involvement.

It appears that both bone marrow aspiration and skeletal roentgenologic survey are of complementary value in the total evaluation of the patient. Berkheiser[196] demonstrated malignant cells in 12 of 246 unselected, consecutive bone marrow aspirations. In a larger series of 1100 consecutive marrow aspirations, Amy and Jaimet[197] report that 24% were positive for malignant cells.

For reasons of ease and safety the iliac crest is preferred for obtaining marrow aspiration. Rubinstein[198] reports that the iliac crest is involved at least as often as other bones, once blood-borne metastasis has occurred.

King[199] has described a method that utilizes TELFA* to concentrate marrow elements.

Procedure—marrow aspiration, smears and sections:

1. Aspirate no more than 2 ml. from the sternum, iliac crest, or other preferred sites into a 20 ml. syringe that has just been rinsed with 10% EDTA (dipotassium ethylenediaminetetraacetate).
2. Immediately disconnect and withdraw the needle.
3. Rotate the syringe to prevent clotting.
4. Place 0.5 ml. of aspirate in a Petri dish and carefully select marrow elements on the end of a wooden applicator stick. Allow fluid blood to soak into the wood for about 30 sec.
5. Gently touch the applicator stick to the surface of precleaned glass slides, making rows of 5 to 6 touch patterns that cover ½ to ¾ of the slide.

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*Curity, Bauer & Black Division, Chicago, Ill.

6. Routine cover slip or slide preparations may be prepared, as desired.
7. Combine the remainder of the material in the Petri dish and syringe and drop into the center of a 2 in. square pad of TELFA. Erythrocytes and serum are rapidly absorbed in the cotton pad, while marrow elements remain on the surface of the TELFA.
8. Separate the TELFA from the pad and place in Bouin fixative for 1 hr.

The marrow elements concentrated on the TELFA form a firm button which can be processed for routine paraffin sectioning.

CANCER CELLS IN THE CIRCULATING BLOOD

Malignant tumor cells were reported in circulating peripheral blood by Thiersch[200] in 1865, by Ward[201] in 1913, and by Pool[202] in 1934. More recently, reports by Engell[203] in 1955 and by Sandberg and Moore[204] in 1957 stimulated the interest of cytologists, clinicians, and surgeons to investigate the frequency with which metastatic cancer could be detected by blood examination and to relate the finding to its prognostic significance.

Engell[203] examined serially sectioned paraffin blocks of blood clots and identified tumor cells in 9.7% of patients with cancer and reported "atypical, tumorlike cells" in an additional 8.5%. Sandberg and Moore[204] precipitated erythrocytes by the addition of fibrinogen. They reported finding tumor cells in 38% of patients with advanced adenocarcinoma of various primary sites.

More methods have recently been described for separating tumor cells from normal hemic elements. Fawcett et al.[205] applied a flotation method for separation of erythrocytes in serous fluid. The method depended upon the difference in specific gravity of various dilutions of bovine albumin in saline. In 1954 McGrew[206] applied the method to isolation of cancer cells in circulating blood. In 1959 Seal[207] reported a modification utilizing a solution of blended silicones (SG 1.075) as a medium for separating erythrocytes and leukocytes under the silicone, and cancer cells and lymphocytes on the surface of the silicone. Seal also employed Millipore filtration for cell collection.

The significance of the presence of malignant cells in circulating blood is not yet clearly defined. The method of study does not appear to be of practical value for detection of early curable cancer, and the presence of tumor cells in circulating blood cannot be interpreted as signifying that metastatic spread has occurred. Engell[208] recently reported a follow-up study on patients in his earlier series who were found to have tumor cells in the circulating blood at the time of surgery. He states that, of the patients who have survived from 5 to 9 yr. after cancer surgery, 51% had tumor cells in the circulating blood at the time of the operation.

Chapter 97

Identification of benign and malignant cells

SAMUEL L. PHARR

Little experience is required to distinguish between typically normal and characteristically malignant cells. In the majority of cases, however, this distinction is not so clearly apparent, and accurate cytologic interpretation requires a careful and experienced appraisal of borderline changes. Thorough familiarity with cellular appearances in each type of secretion must be obtained from a study of healthy individuals as well as from patients with a wide variety of pathologic but benign conditions.

NONMALIGNANT CELLS

Squamous epithelial cells

Squamous epithelium undergoes a continuous process of cell maturation and shedding; thus the secretions that have contact with a squamous epithelial lining (i.e., the oral and nasal cavities, esophagus, vagina, anus, and skin) contain large numbers of exfoliated squamous cells. The maturation pattern differs between skin and mucous membrane, and the influence of hormones or inflammatory processes may alter the maturation process, particularly in the vaginal mucosa.

The generally accepted terminology for the cell forms of squamous epithelium applies the terms "parabasal," "intermediate," and "superficial." It should be kept in mind that ascribing a name to a form of squamous epithelial cell is merely a means of indicating its relative stage of maturity and has no relationship to the identification of a specific cell type.

Basal (squamous epithelial) cells: For practical purposes the basal cells are **not** encountered in cytologic preparations. The basal zone of squamous epithelium rests upon the basement membrane and consists of a single layer of undifferentiated cells which are not ordinarily exfoliated.

Parabasal (squamous epithelial) cells: These are spherical or ovoid cells which measure approximately 15-30μ in diameter and which originate in the deep layers of squamous epithelium directly above the single germinal layer. The cytoplasm is ordinarily basophilic and has a smooth, distinct boundary. Parabasal cells when seen in groups appear polygonal rather than round and may show intercellular bridges. The nucleus is usually single, centrally placed, and of uniformly round dimensions. Fine chromatin granules are evenly distributed throughout the nucleus, and a small nucleolus may be present.

Intermediate (squamous epithelial) cells: These cells are somewhat larger than parabasal cells and range in size approximately from 20 to 40μ in diameter. The increased size results from the swelling and elongation of cells in the intermediate layers of squamous epithelium. Smooth irregularity in the cytoplasmic contour is often present. The cytoplasm of intermediate cells is most often basophilic but may be acidophilic. The nuclei of intermediate cells vary from the size of parabasal nuclei to small, moderately degenerative pyknotic forms; the more usual form lies somewhere in between. When inconsistency between nuclear maturity and cellular flattening is observed, it is advisable to accept the over-all shape of the cell rather than the nuclear characteristics as the most dependable criterion for relating the cell to its appropriate cell layer.

Superficial (squamous epithelial) cells: These are the mature cells that are shed from the outermost few layers of squamous epithelium. In cytologic smear preparations superficial cells are large, often with a diameter of 50-60μ. The thin, filmy, transparent cytoplasm is frequently curled or overturned, and its boundary may be frayed or may blend into the background material. The cytoplasmic staining reactions of superficial squamous epithelial cells provide the artistic play of colors that is characteristic of Papanicolaou-stained smear preparations and may accept a pale basophilic stain or, more frequently, the acidophilic pink, yellow, or orange staining components. Typically, the nucleus of the superficial cells is a small condensed mass of chromatin with an intact nuclear membrane; frequently, however, the nucleus may be absent or karyorrhectic.

Columnar epithelial cells

Respiratory tract: Portions of the nasopharynx and larynx, as well as the entire length of trachea, bronchi, and bronchioles to their terminal portion, are lined by respiratory epithelium. This is composed of tall, pseudostratified, ciliated columnar cells with a varying distribution of nonciliated, secretory goblet cells interspersed. The cells that line the terminal bronchioles are a lower, ciliated cuboidal form; otherwise there are no distinguishing characteristics for identifying the precise area from where columnar cells have been shed. Exfoliated columnar cells vary markedly with their state of preservation, with the method of specimen collection, and as a result of various respiratory diseases. Relatively few columnar cells may be observed in specimens raised spontaneously from the respiratory tract and, when present, often show degenerative changes incurred during their passage and expectoration. Denuded nuclei, as well as swollen or shrunken nucleated forms, may be present within the same specimen. Specimens collected by aspiration or lavage from the respiratory tract, on the other hand, contain well-preserved columnar cells in groups and in single cell distribution.

The nuclei of columnar epithelial cells are basally placed in a clear or finely reticulated cytoplasmic column of variable length and shape. The cytoplasm of ciliated forms tends to conform to the width of the nucleus distally to its ciliated free surface. Variability is seen in the length and the degree of constriction of the proximal extension of cytoplasm by which each cell is attached to the basement membrane. The average diameter of columnar nuclei is approximately 6μ; however, a nuclear diameter of twice this size may be observed in some benign respiratory diseases. In well-preserved forms the chromatin is moderately coarsely granular and evenly distributed throughout the nucleus. The nuclear membrane is prominent and of uniform thickness.

The secretory cells of respiratory columnar epithelium possess no cilia. Cytoplasm is more abundant than in ciliated columnar cells and contains distinct interlacing vacuoles of variable size. The goblet form is usually present,

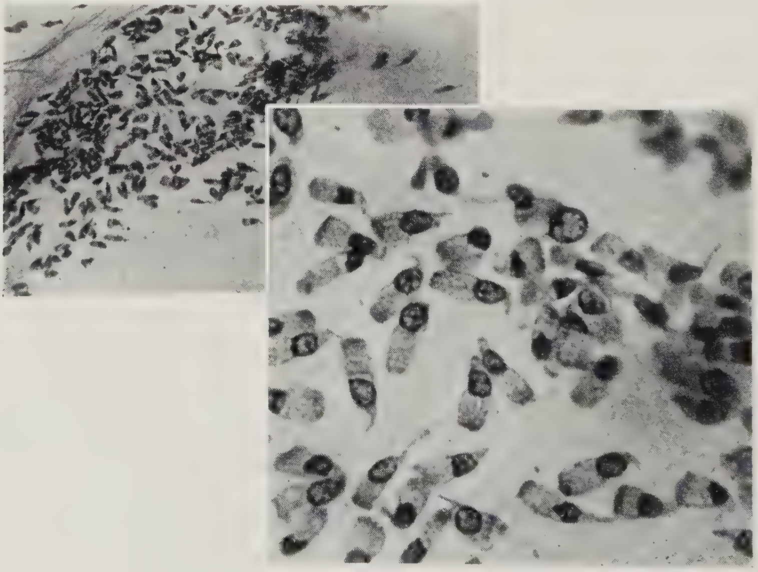

Fig. 609. Typical tall, ciliated, bronchial columnar epithelial cells obtained by bronchoscopic aspiration.

as well as secretory cells which are less distended by mucus. Cytoplasmic boundaries may be frayed or indistinct, and the nucleus is often opaquely pyknotic and compressed peripherally into a triangular shape.

Female genital tract: The abrupt junction of squamous to columnar epithelial lining is somewhat variable in anatomic location but generally occurs at the level of the external os. A second, less striking transition is found in the area of the internal os, marking the junction between endocervix and endometrium. In tissue sections the endometrial mucosal surface is lined by a single layer of tall, clear, secretory columnar epithelial cells with patches of cells possessing cilia. The columnar cells that form the endometrial mucosa are lower, often rounded, and more densely packed in a pseudostratified layer. Small groups of ciliated cells are also scattered among the secretory cells. The nuclei of endometrial cells are less uniform in size than those of endocervical cells and are more hyperchromatic. These histologic differences are reflected to some extent in cytologic appearances and assist in the differential identification of endocervical and endometrial cells. Considerable uncertainty may be experienced, however, in cytologic identification of intermediate forms. Large clusters of endometrial stromal cells are quite characteristic and are present in smears collected during the first 10 or 12 days of the cycle. The oviducts are similarly lined by secretory and ciliated columnar cells; however, they lack distinctive characteristics to permit precise identification in cytologic smear preparations.

Gastrointestinal tract: The transition between the stratified squamous epithelial tissue of the esophagus and the columnar epithelium of the stomach occurs abruptly at the cardiac junction. The surface epithelium of the stomach is composed of a single layer of tall, uniform columnar cells. No ciliated epithelium is present throughout the gastrointestinal tract. Cytologic specimens obtained from the stomach by aspiration of contents or by lavage may contain relatively few gastric mucosal cells. Sheets of gastric columnar cells may be seen in specimens obtained by forceful lavage or by abrasive instrumentation, and in smears they are often seen *en face* in honeycomb arrangement. The cytoplasm is delicately reticulated and is stained faintly yellow or yellow-pink in the Papanicolaou method. Single gastric mucosal cells may be degenerative as a result of partial digestion by gastric secretion. Cells from the upper and lower respiratory tract may be deposited in the stomach through swallowing and can be recovered in the cytologic gastric specimen.

The distinctive cell forms that line the gastric glands (zymogenic, parietal, mucous neck, and argentaffin cells) cannot be identified with certainty in cytologic smear specimens. Occasionally cells with intensely orangeophilic cytoplasmic granules are seen which are suggestive of Paneth cells; their presence in gastric secretion may result from intestinalization of gastric mucosa.

Cells collected from the mucosal surface of the duodenum are essentially identical to those of the gastric mucosa. At present (1963), the cytologic method is not applicable for studies of the jejunum and ileum.

The epithelial lining of the colon and rectum is composed of uniform secretory columnar cells with many goblet cells interspersed. There is little difficulty in identifying normal epithelial cells because of their characteristic uniformity; however, there is often a paucity of well-preserved cells.

Transitional epithelium; urinary tract

The urethra, bladder, ureters, and renal pelvis are lined by a somewhat specialized stratified surface known as transitional epithelium. As implied by the term, the epithelial components are intermediate between stratified squamous and simple columnar epithelium. Transitional epithelial cells in cytologic preparations are characterized by marked variation in size and form and range from typical parabasal squamous to typical simple columnar type. The intermediate forms are often strikingly enlarged, with a proportional enlargement of nucleus. Multinucleation is frequently present. The added pleomorphism resulting from cellular changes in benign pathologic conditions makes this area one of the most challenging for cytologic examination.

Mesothelium

The pleural, peritoneal, and pericardial cavities and the synovial cavities of the joints are lined by a single-layered mesothelium. Under normal circumstances these spaces are moistened by a thin layer of serous fluid in insufficient quantity to collect for cytologic examination. It is not meaningful, therefore, to describe the normal appearance of exfoliated mesothelial cells. The complexity of cellular appearances attendant to the accumulation of fluid in benign pathologic conditions precludes inclusion of a generalized morphologic description of cellular appearances here.

Reticuloendothelial cells

Wandering phagocytic cells of the reticuloendothelial (RE) system are a constant finding in all types of specimens examined by the cytologic method. They are perhaps the most variable morphologic forms which are encountered. Their number and morphologic features vary from site to site and in the presence of various pathologic conditions. Although purely academic, it may be impossible at times to distinguish between atypical forms of histiocytes and parabasal squamous epithelial cells. More importantly, bizarre histiocytes frequently present characteristics that may closely resemble those found in malignancy. It is indeed fortunate for the cytologist that RE cells occur and function as single cells, thus directing conservative evaluation of abnormalities observed in single cells, when no groups with similar changes can be found.

A generalized description of RE cells is of little benefit for identifying unusual forms. The nucleus is most frequently eccentric; multinucleation, lobulation, and constrictive nuclear distortion are commonly observed. The cell size and cytoplasmic structure of RE cells often reflect their function, environment, and state of activity; thus a variety of terms are applied—i.e., monocyte (blood-borne), histiocyte (in tissue), macrophage (free in secretion), lipophage (containing fat inclusions), and histiocytic syncytium (an elaborately multinucleated RE cell).

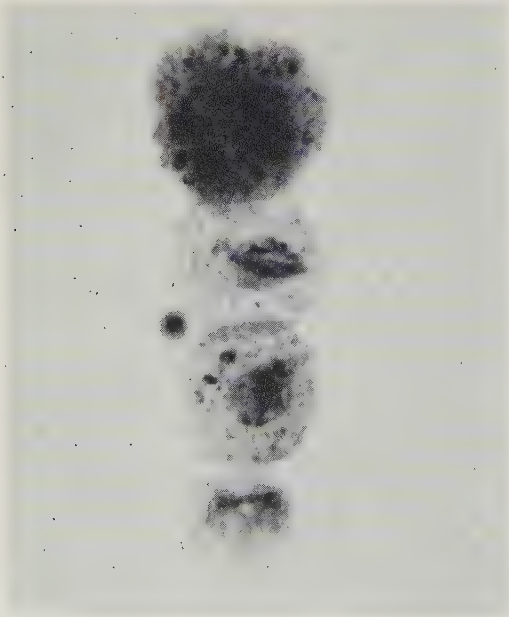

Fig. 610. Pulmonary macrophages in sputum. Note that uppermost cell is filled with phagocytosed material.

Miscellaneous benign cells and structures

Polymorphonuclear leukocytes (including eosinophils), erythrocytes, lymphocytes, monocytes, and plasma cells are recognized in cytologic smear preparations, but they are less specifically stained by the Papanicolaou procedure than by conventional hematologic staining methods. The proportion and types of blood elements offer a useful clinical indication of the status and degree of infection. In addition, the consistency in size of blood elements provides the cytologist with a convenient and familiar reference point for evaluating cell size. Staining intensity of leukocyte nuclei is often used to appraise the over-all nuclear staining quality in cytologic smears.

Bacteria and fungi are easily recognizable in cytologic smears, but more appropriate methods of study are usually required for precise identification. *Trichomonas vaginalis* and *Candida albicans* can be readily identified in Papanicolaou-stained vaginal smears, and to a more limited extent cytologic examination of smears of various secretions may disclose other parasites or their ova.

CYTOLOGIC IDENTIFICATION OF MALIGNANCY

Malignancies that arise in an epithelial surface can be detected at an early stage by the cytologic method; thus diagnostic cytology is primarily applied to detection of epithelial malignant tumors—carcinomas. At present (1963), utilization of the cytologic method is quite limited for the detection of malignant tumors that arise in the underlying connective tissue, muscle, bone, or in other areas where erosion into a draining passage occurs late in development (lymphoma, sarcoma, etc.).

The variety of cellular appearances in carcinoma is infinite, and the possible range within a single tumor extends from cells that cannot be distinguished from the typical normal cells of the organ to tumor cells whose changes exceed the imagination. It is not possible to describe or to identify a cancer cell as a specific entity. A cytologic opinion of malignancy results from an accumulation of definable cellular and nuclear characteristics which are known to be present in proved cases of cancer. The categorized features of malignant cells are seldom, if ever, diagnostic as a single observed entity. For example, marked nuclear enlargement per se can be a meaningless finding in the absence of additional nuclear structural abnormalities of chromatin pattern, nucleolus, or nuclear membrane. Likewise, observation of a single abnormal cell possessing abundant evidence of malignancy requires conservative judgment if no supportive evidence of cellular abnormalities can be found in the same specimen.

Criteria of malignancy

Nuclear abnormalities

Cytologic evidence of malignancy is derived almost entirely from observations of **nuclear** abnormalities. Changes in the nucleus of a cell must always, however, be appraised in relationship to its cytoplasm and particularly with regard to the interrelationships of adjoining cell nuclei within a group.

Nuclear hypertrophy: Increase in nuclear size indicates that a relative relationship must be established. In the case of well-differentiated tumors the nuclear volume of its component cells can be compared with the normal nuclear size seen in its benign counterpart. In undifferentiated or poorly differentiated malignant forms there may be no relationship to benign cell types; thus significance must be applied to an arbitrary nuclear diameter. A nuclear diameter greater than 20μ can be

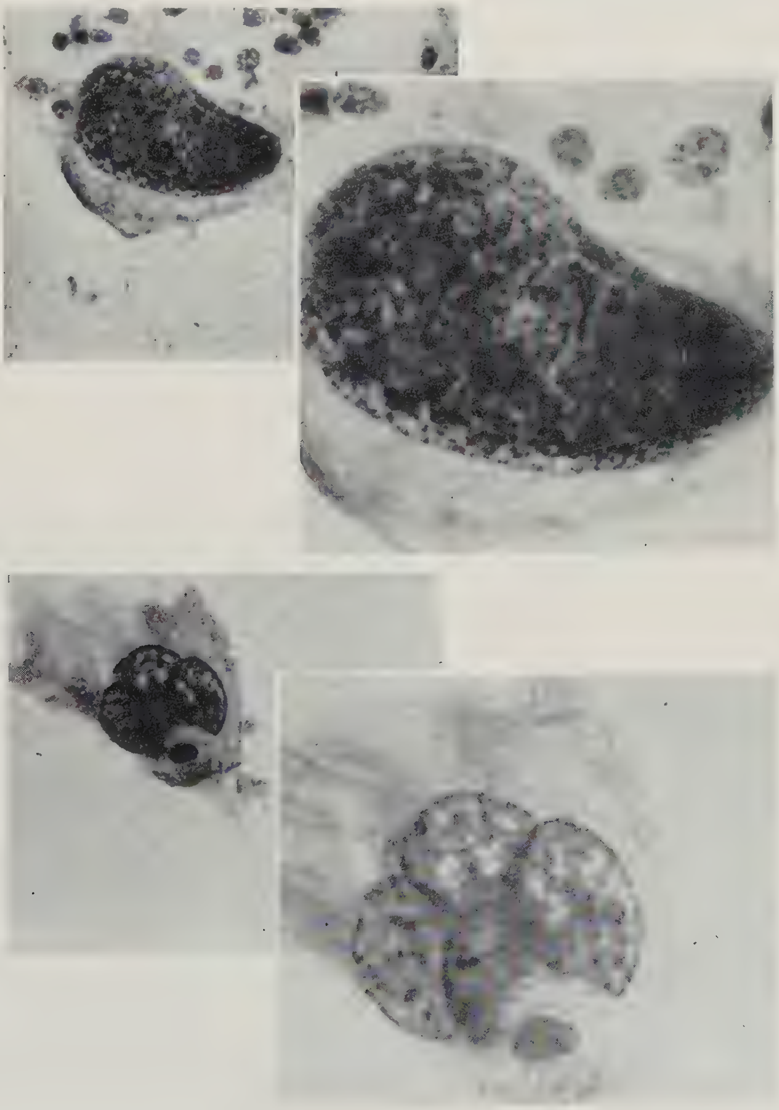

Fig. 611. Examples of single, malignant cells. Above, cells from pleural effusion. Below, cells from sputum. Note enlarged nuclei with dense, irregular chromatin.

interpreted as presumptive evidence of abnormality but is not an a priori indication of malignancy. Although nuclear hypertrophy is a frequent manifestation of cancer, it must be emphasized that nuclear diameters greater than 20μ are observed in benign cells at times and that in some cases malignant cell nuclei may be ½ this size.

Chromatin abnormality: Two forms of chromatin abnormality may be present in malignant cells: hyperchromasia and irregular distribution.

Hyperchromasia: This is a frequent manifestation of malignancy and reflects an increase in the nuclear content of deoxyribonucleic acid (DNA). Nuclear hyperchromatism and hypertrophy are usually companion abnormalities in malignant cells, and these characteristics provide the basis for successful screening of cytologic smears at low-power magnification (10×). The significance of hyperchromasia is also relative to other findings, and it must be compared to the over-all staining intensity of familiar cell nuclei.

Irregular distribution: The pattern of chromatin distribution within the nucleus provides the most dependable **single** criterion for identification of malignant cells and is particularly significant when there is also an increase in nuclear size and in total chromatin content. Abnormality in chromatin pattern within a malignant cell nucleus is frequently characterized by massive condensation and irregular distribution throughout the nucleus. Areas of clearing, formed by chromatin clumping, assist in distinguishing hyperchromasia from complete nuclear opacity resulting from overstaining or advanced nuclear degeneration.

Nuclear shape: The shapes of malignant cell nuclei often depart from the usual round or oval forms of benign epithelial cells. Jagged, sharp protrusions of nuclear contour are quite suggestive of malignancy when present in enlarged nuclei with chromatin abnormalities. Lobulation, pseudopod projections, or smooth constrictive irregularities of the nuclear contour may also be significant observations. Prominence of the nuclear membrane, particularly when irregularity in thickness is present, is an important consideration.

Multinucleation: Multinucleation can be interpreted as supportive evidence of malignancy only when the nuclei are of abnormal size and structure. Anisonucleosis of multinucleated cells further strengthens the opinion of malignancy.

Mitoses: Although an increase in mitotic activity is a frequently observed characteristic present in histologic sections of malignant tumors, mitotic cells are rarely encountered in cytologic smear preparations (with the exception of serous fluids, where they may be abundant in **both** benign and malignant conditions). The paucity of mitotic cells in exfoliated secretions appears to be due to the lack of dividing forms in the superficial desquamating tumor surface. Demonstration of mitotic figures and particularly "abnormal mitoses" in secretions other than serous fluids can provide strong supportive evidence of malignancy. However, it must be related to other cellular abnormalities present in the same specimen.

Cytoplasmic abnormalities

Nucleus-cytoplasm ratio: The relationship of nuclear volume to total cell volume, like nuclear hypertrophy, is a relative appraisal. For differentiated malignant cells a relationship is established to the nucleus/cytoplasm (N/C) ratio observed in the corresponding benign counterparts. In well-differentiated, keratinized malignant squamous cells the volume of cytoplasm may exceed that of the nucleus and yet be significantly altered, since only a small pyknotic nucleus is normally present in a benign, keratinized squamous cell. Likewise, cytoplasmic volume of hypervacuolated adenocarcinoma cells may greatly surpass nuclear volume. In less differentiated

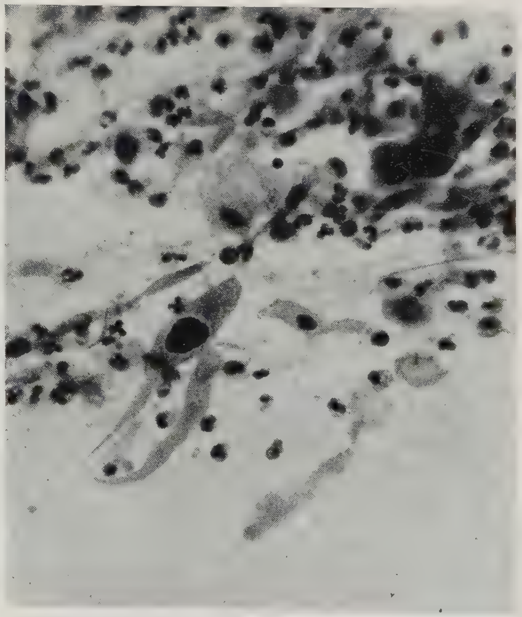

Fig. 612. Malignant "tadpole" cell in vaginal smear from patient with squamous carcinoma of cervix.

malignant cells, however, this ratio is generally reversed, with nuclear predominance.

Differentiation: Identification of tumor cell type stems from a critical appraisal of cytoplasmic structure and form. It must be emphasized that identification of tumor cell type cannot be made in every case and that it should always be a **secondary** step following the initial identification of malignancy based on nuclear abnormalities.

Squamous carcinoma: Hyperkeratinization of cytoplasm is regularly associated with well-differentiated squamous cell tumors and can be somewhat specifically identified in Papanicolaou-stained smear preparations by a brilliant orange, granular staining reaction. In addition to malignant nuclear changes seen in hyperkeratinized cells, a number of bizarre cytoplasmic shapes may be present, including the so-called spindle and tadpole forms. These pleomorphic cells may or may not be keratinized and often contain only minimal nuclear evidences of malignancy. Observation of such forms provides strong presumptive evidence of

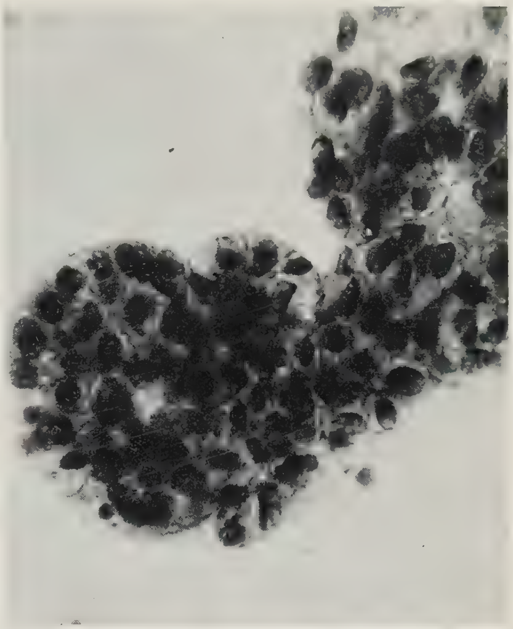

Fig. 613. Hypervacuolated malignant cells which form a glandular structure seen in vaginal smear from patient with endometrial adenocarcinoma.

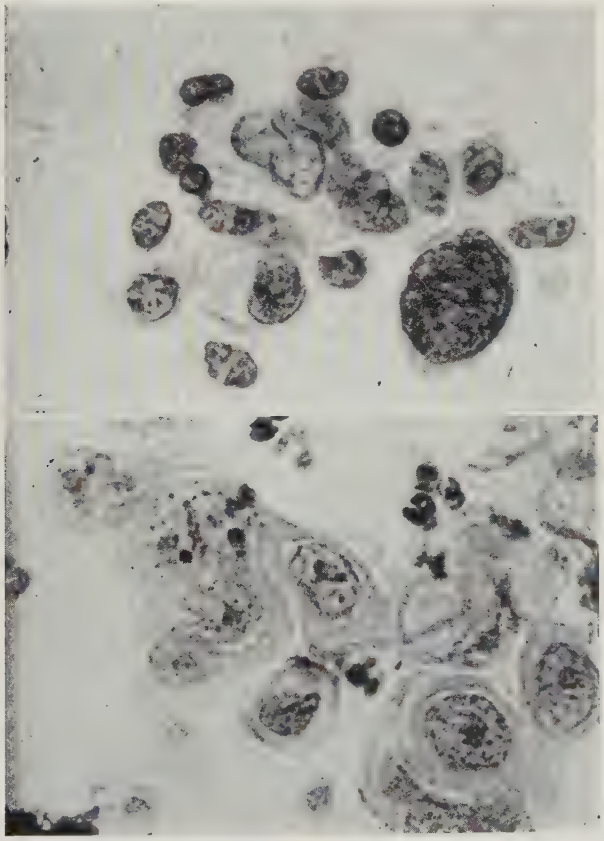

Fig. 614. Groups of malignant cells. Above, cells from cervical smear. Below, group of cells from urine.

squamous cell carcinoma only after malignancy has been established upon firm nuclear grounds.

Adenocarcinoma: Adenocarcinoma is evidenced by demonstration of hypervacuolated cytoplasm in malignant cells which form a glandular, unit structure—an **acinus.** Vacuolization within a single cell, regardless of its extensiveness, cannot be interpreted as evidence of adenocarcinoma. Multiple vacuoles are seen in adenocarcinoma cells and are often of enormous size. Distinct septae are present, and the nucleus is usually distended to the cell periphery.

Abnormalities in cell groups

The decrease in mutual adhesiveness of malignant tumor cells produces abundant shedding and provides the most important basis for conducting diagnostic cytologic examinations. At times, however, fragmentation of tumor may extend to its individual cellular components, and thus cytologic identification of malignancy may be made difficult when there is a predominance of single tumor cells. This is particularly true in cytologic specimens from well-differentiated squamous cell carcinoma. Although it is not necessary to observe a large number of groups of malignant cells for a positive cytologic diagnosis, at least a few groups of joined malignant cells must be seen so that abnormality in intercellular relationships can be evaluated. These abnormalities consist of marked variation in nuclear size and shape, discrepancy in maturation, and changes in polarity and parallelism.

Marked variation in nuclear size and shape: Anisonucleosis is a valid indication of malignancy only when the artifactual effect of nuclear shrinkage or swelling can be ruled out; thus for significance the chromatin pattern of adjoining nuclei must be similarly preserved. The nuclei of adenocarcinoma and undifferentiated cell groups tend to vary in size more than in shape, whereas those of squamous cell carcinoma vary in both size and shape.

Discrepancy in maturation: The tendency of normal epithelial tissue to mature at a rate that is uniform among neighboring cells is frequently lost or altered in epithelial malignant tumors. Abnormality of growth rate may also be observed in benign epithelium undergoing repair. Inequality in the rate of maturity can be assessed only in groups of cells that are distinctly joined to one another. The abnormality is characterized by variation in the degree of cytoplasmic maturation observed within a cell group. Discrepancy in maturation is most apparent in squamous cell carcinoma groups in which hyperkeratinized (orangeophilic) malignant cells are joined to immature (basophilic) and intermediate malignant cells. The significance of this finding depends upon the degree of accompanying nuclear abnormality.

Changes in polarity and parallelism: The tendency of malignant tumors to proliferate wildly and without the restraining influence of normal, orderly growth is most apparent in histologic sections. Unrestrained and disoriented growth pattern may be suggested in cytologic smear preparations, but the terms "polarity" and "parallelism" cannot be applied with accuracy, since there is no basement membrane or a similar fixed tissue structure from which to relate the direction and pattern of growth. The most convincing cytologic evidences of malignancy occur as a direct result of abnormal growth pattern and are reflected in the crowded positioning of malignant cells within a group. Mutual cellular and nuclear distortion results, and there is an impression of a loss of parallelism. Since crowding may result from mechanical distortion in smear preparation, the primary judgments of malignancy must be based on abnormalities of nuclear structure.

The importance that is contributed by finding groups of malignant cells cannot be minimized; it must be pointed out, however, that one of the most frequent sources of false positive cytologic diagnosis has resulted from placing a disproportionate emphasis upon structural characteristics present in cell groups, without first satisfying the **nuclear** requirements for a cytologic diagnosis of malignancy.

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PART VI

CLINICAL MICROSCOPY

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Section One

URINE ANALYSIS

Urine is an extremely complex solution of organic and inorganic substances offering a most convenient source of information which may be obtained quickly and inexpensively. As a result urine analysis is of basic diagnostic importance and an integral part of the examination of patients in all branches of medicine.

For the most part the constituents of urine are waste products of various metabolic processes and of ingested foods. Excessively large or small quantities of normally occurring substances or the presence of materials ordinarily absent in specimens of healthy subjects are often significant clues in the process of making a diagnosis or of following a therapeutic regimen.

Routine urine analysis generally refers to the determinations of specific gravity and acidity, the presence or absence of protein, sugar, and acetone, and a microscopic examination of the solid material. All other tests are considered to be more or less special, although some of these are performed so frequently that they too are essentially part of the routine analysis.

In the following chapters information is presented that covers all of the usually requested assays and, in addition, many of those that are requested rather infrequently. In keeping with the tradition established by the past editions of this text, material difficult to find elsewhere is included.

Chapter 98

General considerations

SAM FRANKEL

URINE PRESERVATIVES

It seems appropriate to discuss the various means available for preserving urine specimens at the beginning of the discussion since the quality of many of the subsequently described procedures is entirely dependent upon the state of the sample. Several potential changes may occur with improper preservation. The action of bacteria or mold spores will convert urea to ammonia, thereby altering the pH as well as the nitrogenous constituents. Glycolysis will cause loss of glucose and at the same time will increase acidity, thus invalidating the pH determination. Accompanying changes in pH will be precipitation of certain constituents, such as phosphates in alkaline solutions and urates in acid solutions.

Also to be considered are the constituents to be assayed, for in many cases a preservative suitable for some materials will interfere with others. A further complication is due to the various procedures available for the same type of assay since a preservative may interfere with one method but not with another.

Whenever possible the specimen should be freshly voided and immediately processed. For most practical purposes, however, there is always a time lapse before analysis is performed.

For routine urinalysis specimens are seldom collected under sterile conditions. Most preservatives are therefore primarily for the purpose of inhibiting bacterial or mold growth. Although some of the commonly used preservatives are most effective against contamination acquired after the specimen has been voided, it is good practice to provide some means of immediately closing the container.

Whether or not preservatives have been added to the empty container before collection, the specimen should be immediately protected by storing at cool temperatures. The drop in temperature will often cause certain constituents to precipitate and will also affect pH and specific gravity; therefore, rewarming to room temperature and thorough mixing should be part of the standard procedure.

The types of materials that have been recommended vary considerably and are often designed for the protection of a single constituent. Although a universal preservative has long been sought, no such material is yet available. Several suggested compositions, although suitable for routine analysis, do not apply in the case of special assays or for certain specimens containing unusual quantities of pathologic constituents.

Perhaps the most commonly used preservative is **toluol.** In this case a thin film of toluol floats on the surface of the specimen and is satisfactory for keeping out contamination although it is not very effective against bacteria or mold spores which are already in the sample. Another objection to toluol is that it enters pipets and has a certain nuisance value. This can be overcome by taking the bottom portion of the specimen contained in a separatory funnel although the time factor and lack of sufficient equipment would be a disadvantage when a large number of specimens are handled.

Chloroform, also frequently used as a preservative, has the advantage of producing a saturated solution so that it does inhibit growth in the specimen, but it does not form a surface layer so that contamination from outside is not prevented. Furthermore, chloroform has been reported to interfere with some of the tests for glucose such as the Fehling reduction test. **Formalin** also has the same advantages and disadvantages.

For 24 hr. urine collections, especially those to be assayed for ammonia, urea, or total nitrogen, a suitable preservative is 10 ml. concentrated hydrochloric acid. In this case, however, uric acid may be precipitated so that thorough mixing is required before this assay is performed.

For urine specimens that are being collected specifically for glucose analysis, such as in a glucose tolerance test or as an aid in regulating the insulin dosage of a diabetic, sodium fluoride or benzoic acid will inhibit glycolysis.

SPECIMEN COLLECTION

The first requirement for the collection of a valid specimen is a clean container that can be closed. Frequently the lids provided with many of the commercially available disposable containers are not used, thereby considerably increasing the chances of contamination.

In all cases, except for routine analysis, specimens should be collected at timed intervals. Because of variations in the excretion of various materials from one part of the day to the next, a 24 hr. specimen is necessary for any quantitative study. In certain cases, such as for clearances or enzyme excretion tests, timed specimens of 1 or 2 hr. are required. In other cases longer times are recommended. In every case, however, when quantitation is involved, time control is essential, and extreme care should be taken to guard against the loss of any portion of the sample.

EXAMINATION

Volume: Volume in 24 hr. is measured by pouring the urine into a 1000 ml. cylinder and reading the number of milliliters present. The **normal volume** of urine in 24 hr. is 750-2000 ml. or 25-65 oz. The average is 1500 ml.

Color: Record the color of the urine, for example, very pale yellow, light amber, dark amber, greenish, or brown. The color of the urine is dependent upon many factors. Normally, it is yellow or reddish yellow due to the presence of several pigments, especially urochrome. The intensity of the color depends upon the concentration of the urine. Dilute urines are usually pale, whereas concentrated urines are darker. Acid urines are usually darker than alkaline urines. The color may be greatly changed by the presence of abnormal pigments and by various drugs, as follows:

Blood: red or brown; smoky
Bile: yellowish or brown, turning greenish; yellow foam
Chyle: milky
Methylene blue: greenish blue
Phenols: olive green to brownish black, etc.

The color of urine is often affected by drugs, as follows:

Dark green: phenol, phenyl salicylate, creosote, and resorcinol
Red: antipyrine and trional (sulfonethylmethane)
Brown or black: pyrogallol
Yellow: phenacetin and picric acid; santonin changes from bright yellow to scarlet with the addition of sodium hydroxide

Red urine is also caused by eating beets.

Odor: Record as normal, aromatic, medicated, ammoniacal, putrid, strong.

Transparency: Record as clear, cloudy, turbid, or cloudy with sediment.

Freshly voided normal urine is clear or transparent but may be cloudy as a result of the presence of phosphates and pus. The phosphates disappear upon the addition of acid; pus does not disappear but may become gelatinous. Freshly voided urine may be cloudy from the presence of bacteria or may be comparatively clear with numerous shreds of mucopurulent material from chronic urethritis.

The transparency record is a factor only in dealing with fresh specimens because all specimens become cloudy due to bacterial growth and alkaline salt formations upon standing.

On cooling and standing, all specimens develop a faint cloud of mucus, leukocytes, and epithelial cells which settle to the bottom—the so-called nubecula. Acid urines may develop a white or pinkish sediment of amorphous urates, whereas alkaline urines may develop a heavy white sediment of amorphous phosphates. Urine containing pus shows a heavy mucoid, whitish sediment. Blood gives a reddish brown smoky sediment. Uniform cloudiness which cannot be removed by ordinary paper filtration is due to the presence of bacteria. Turbidity of urine is often due to the presence of fat or of spermatozoa.

REACTION

Dip a piece of blue litmus paper into the urine. If it turns pink, the urine is acid. If it does not change color, place a piece of red litmus paper in the urine. If it turns blue, the urine is alkaline. If neither the blue nor the red paper changes color, the urine is neutral. If the red litmus turns blue and the blue litmus turns red, the reaction is amphoteric.

Normal urine is acid in reaction with a pH ranging from 4.8 to 7.5 or a general average of 6.0. Urine may be neutral or alkaline as a result of the administration of alkalies. Diet may influence the reaction. Acid urine is usually present in acidosis, diabetes, starvation, diarrhea, etc. Alkaline urine occurs in alkalosis, severe vomiting, urinary infections, etc.

Determination of hydrogen ion concentration

The hydrogen ion concentration is determined with pH paper or with indicator solutions. For this method use fresh clear urine. Use the method outlined on pp. 20 and 21, and also see potentiometer measurement of the pH on pp. 22ff.

Nitrazine indicator for use in acidimetry and alkalimetry

Nitrazine* is a very sensitive indicator for use in acidimetry and alkalimetry, both for titration purposes and as an indicator of hydrogen ion concentration. It is especially useful in determining the reaction of urine, saliva, and other body fluids. The composition of food is perhaps the most important factor in determining the reaction of urine. The so-called alkaline tide occurs shortly after eating because of the increased alkalinity of the body fluids subsequent to the loss of acid for purposes of digestion.

Urine that has stood for some time becomes alkaline to litmus as a result of ammonia production due to the action of bacteria on urea. The alkaline reaction of urine of patients on a vegetable diet is due to the fact that vegetables and fruits, including acid fruits, contain acid salts of dibasic or other organic acids. These acids are converted to carbonates and have an alkaline reaction because they form some free alkali by hydrolysis. Part of the carbonic acid is lost to the body through the lungs, leaving the associated strong base together with some of the very weak carbonic acid to find their way into the urine.

Alkalinity of the urine can be raised by the ingestion of sodium bicarbonate.

Nitrazine changes color within approximately the same range as litmus, but since the indicator is more sensitive and the color changes are much more definite, differential readings can be made over smaller pH intervals.

Another advantage of Nitrazine is that the readings are made within 1 or 2 min. after applying a drop of the solution to the paper. When the Nitrazine test solution is used, the reaction is immediate and permanent.

The color values on Nitrazine paper observed when read in 60-120 sec., with the solution applied with glass rod, are as follows:

pH 4.0	bright yellow
pH 4.5	yellow, slightly duller (lemon yellow)
pH 5.0	mustard yellow
pH 5.3	light olive yellow
pH 5.5	olive yellow
pH 5.7	olive green with yellow cast
pH 6.0	olive green
pH 6.2	bluish cast to olive green
pH 6.4	slightly stronger bluish cast to olive green
pH 6.5	dark green
pH 6.6	blue gray
pH 6.8	dark blue gray
pH 7.0	gray blue
pH 7.3	deeper blue
pH 7.6	same
pH 8.0	same

Hydrion pH test papers

Hydrion wide-range pH test paper* is another method of determination of reactions. There are 5 types of Hydrion paper, showing changes at the following pH values: Hydrion A, pH 2-4-6-8-10; Hydrion B, pH 1-3-5-7-9-11; Hydrion C, 11-12; Hydrion D, 12-13; and Hydrion E, 13-14. These papers are dispensed in rolls in a special container for turning out a piece of the paper when its use is indicated. The paper is also furnished in strips in a vial.

Hydrion pH buffers are also supplied in vials. Each buffer when dissolved in 100 ml. distilled water gives a solution of the indicated pH. The manufacturer supplies the complete set of 55 standards in a permanent wooden case, pH 1.2-12.0 in steps of 0.2 pH unit.

Anachemia papers

A variety of test papers† is available in a wide range type from pH 1.0 to pH 12.0 as well as an acid range of pH 0.0 to pH 5.0 and alkaline to pH 14.0. In addition, 20 fractional range types are made, thus making available an indicator allowing accuracy to within 0.3-0.1 pH unit by use of overlapping papers. (See p. 1837 for combination of pH with sugar and protein tests.)

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*E. R. Squibb & Sons, Division of Olin Mathieson Chemical Corp, New York, N. Y.

*Hydrion reagents are manufactured by Micro Essential Laboratory, Brooklyn, N. Y., and may be procured from Cargille Scientific Inc., New York, N. Y., and Aloe Scientific, St. Louis, Mo.

†Anachemia, Ltd., Montreal, Canada.

TITRATABLE ACIDITY OF URINE IN TERMS OF N/10 SODIUM HYDROXIDE

Folin method

Procedure:

1. Place 10 ml. urine in a beaker.
2. Add 6 gm. finely pulverized neutral potassium oxalate and 1-2 drops of phenolphthalein indicator.
3. Shake the mixture vigorously for 1-2 min.
4. Titrate with N/10 sodium hydroxide solution from a buret until the solution turns a faint permanent pink.
5. Read from the buret the number of ml. of N/10 sodium hydroxide used.

Calculation: Assume the vol. 24 hr. specimen is 1200 ml. and the number of ml. of N/10 NaOH used is 6. A 10 ml. aliquot of the specimen was titrated.

Titer × N × 24 hr. vol./aliquot = mEq./24 hr.
6 × 0.1 × 1200/10 = 72 mEq./24 hr.

SPECIFIC GRAVITY

Consult pp. 1893-1897 for concentration and dilution tests which utilize specific gravity determinations.

Pour the urine into a cylinder. Place a urinometer in the urine, spinning to prevent its floating to the side. Read the specific gravity from the stem of the urinometer.

The urinometer is always calibrated for use at a certain temperature. If the specific gravity is taken at any other temperature, one unit of the last order must be added to the reading for every 3° above the normal temperature, and subtracted for every 3° below the normal temperature.

Example: The urinometer is calibrated for a temperature of 15° C. The specific gravity of the urine at 18° C. was 1.022. True specific gravity is 1.022 + 0.001 or 1.023.

In the event that there is an insufficient amount of specimen, 1 ml. may be weighed on an analytical balance. At normal room temperature the weight of 1 ml. is sufficiently close to specific gravity to be acceptable.

The **normal specific gravity** of the first morning specimen is from 1.015 to 1.020. In diseased conditions it may vary from 1.001 to 1.060. The Exton* immiscible balance is useful to determine the specific gravity of drops of urine when only very small amounts are available, as in ureteral catheterization. The method and construction of the instrument are based on the principle of suspending the urine in an immiscible medium of the same specific gravity which is then determined by the usual methods, making possible rapid manipulations with minimal loss of material. A cylindrical mixing chamber is partially filled with a mixture of petroleum ether and carbon tetrachloride with a specific gravity of about 1.012. The side thistle tube is filled with petroleum-ether and the carbon tetrachloride is kept at hand in a drop bottle. A drop of urine is immersed in the mixture, which is then easily and rapidly varied by means of the stopcock and dropper, so that the urine remains suspended near the middle. The specific gravity of the mixture is then taken by means of the Exton hydrometer; this gives the specific gravity of the urine.

SOLIDS

The amount of solids in 1000 ml. may be roughly calculated by means of the Long coefficient, which is 2.6. Multiply the last two figures of the specific gravity at 15° C. by 2.6 to obtain the solids.

Example:
Specific gravity at 15° C. was 1.016.
16 × 2.6 = 41.6 gm. in 1000 ml. urine.

The output of a normal adult is about 60 gm. It must be remembered that the output of urinary solids is influenced by body weight, diet, exercise, age, metabolism, and state of kidney function.

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*Emil Greiner Co., New York, N. Y.

Chapter 99

Chemical examination

SAM FRANKEL

SUGAR IN URINE

Glucose or dextrose exists in every normal urine in small quantity. It is assumed, however, in ordinary laboratory tests that the presence of sugar in the urine is a pathologic condition or the result of disturbed physiology.

At times other monosaccharides are found, such as fructose, pentose, and galactose. Also of interest are certain disaccharides which consist of 1 molecule of glucose (dextrose) and 1 molecule of either glucose or some other hexose, e.g., lactose, which consists of 1 molecule of glucose and 1 molecule of galactose. Different sugars in urine have different clinical significance.

A large sugar content in urine causes a high specific gravity, even though the color of the urine may be light and the daily output increased.

Pathologically, sugar is present in the urine in undue quantity. This condition is called glycosuria. It is a common incident in the disease known as diabetes mellitus. Glycosuria is also found in other diseases, e.g., in connection with certain poisons, such as morphine, carbon monoxide, mercuric chloride, etc. After prolonged hunger, glucose has been found in the urine.

Alimentary glycosuria: Alimentary glycosuria is a condition caused by undue ingestion of sugar and other carbohydrates by healthy individuals. Excessive ingestion of glucose may so overload the blood stream with blood sugar that the threshold point may be reached.

Renal glycosuria: Renal glycosuria has at times been called renal diabetes. In this condition there is usually a pathologic amount of sugar in the urine without any corresponding rise in blood sugar such as is seen in diabetes mellitus. Furthermore, there are no apparent symptoms of diabetes mellitus.

The following standards have been established in making the diagnosis:

1. Fasting blood sugar is within normal limits, and a normal glucose tolerance curve is expected.
2. Glucose should be present in appreciable quantity in all urine specimens, whether voided in the fasting state or postprandially.
3. Carbohydrate utilization should be normal, as evidenced by the respiratory quotient and serum inorganic phosphate after glucose ingestion.
4. Fat metabolism should be normal, ketosis being more likely to develop during fasting rather than after overeating.
5. Moderate doses of insulin should have little or no effect on the glycosuria.

Bland[1] reviewed the literature and reported 4 cases that conformed in every particular. He called attention to the fact that many persons with melituria of some type are treated for diabetes mellitus and that real damage may be done by such treatment. Since these patients are losing sugar steadily in the urine, it would seem only sensible that a liberal carbohydrate diet is indicated. Certainly, insulin treatment can be of no value in the treatment of renal glycosuria and may do considerable harm. These patients frequently complain of ease of fatigue and malaise, which symptoms may well have their origin in a low carbohydrate intake.

Renal glycosuria is characterized by a low renal threshold for sugar. Urinary sugar is present because of incomplete reabsorption of sugar by the proximal convoluted tubules of the kidneys. The reason for this abnormality is not well understood, although it is known that certain drugs, such as phlorhizin, will interfere with the phosphorylation mechanism known to be present in the tubules. All of Bland's cases had low renal thresholds as proved by the presence of glycosuria with a normal or subnormal level of blood sugar. It has been postulated that a deficiency of tissue phosphatase may be the abnormality producing consequent interference with the phosphorylation mechanism in the renal tubules on which sugar reabsorption depends. Another possible etiology was that suggested by Thomas and Southworth,[2] who conjectured that the renal threshold is under hormone control. Comparison is made with diabetes insipidus in which the hormone of the posterior pituitary exerts its well-known effect on the renal tubules. The renal glycosuria of pregnancy fits this conception in that there exists in pregnancy a hormonal imbalance. In Bland's second case the renal glycosuria developed after a series of streptococcal infections. He suggested that this may be more than coincidental. The widespread vascular lesions apparently produced by repeated hemolytic streptococcal infections may well produce changes in the blood supply to the renal tubules, which, in turn, could alter the tubular physiologic function.

In reference to treatment, a liberal and varied diet should be given with perhaps some emphasis on carbohydrate intake. The patient should be advised that his condition is benign and be reassured that his prognosis is good. Insulin is certianly not indicated. Somogyi[3] has shown that a low carbohydrate intake and ill-advised use of insulin may produce increased hepatic glycogenolysis with each episode of hypoglycemia, thus leading to increased hepatic fat and protein metabolism, with consequent increase in ketone production. Thus, clinical ketonuria may be induced in the absence of glycosuria. In such an instance insulin is clearly contraindicated.

Bland's study included determinations of both arterial and venous blood sugars in the glucose tolerance tests in order to prove adequate removal of glucose by the tissues from the arterial blood. Serum phosphorus determinations were simultaneously made to study the absorption of the glucose and the phosphorylation mechanism.

Pentosuria: In this condition there is a constant excretion of pentose in the urine regardless of the diet. In pentosuria it is believed that there is a disturbance in metabolism and that it is not a precursor of diabetes mellitus. It is essential to differentiate pentosuria from glycosuria because the pentosuric patient must not be subjected to the rigid dietetic control or insulin treatment of the diabetic patient. Tests to be described later (p. 1815) serve to differentiate pentosuria from glycosuria and fructosuria.

An abundance of levulose or fructose is noted at times. These cases of levulosuria or fructosuria are seen after the ingestion of large amounts of fruits. In other words, it is an alimentary condition rather than a metabolic condition. Tests later to be described serve to differentiate fructose from glucose. The Seliwanoff test (p. 1815) is the best test for this purpose.

Alkaptonuria: Alkaptonuria is a condition in which homogentisic acid is present in the urine. In this condition the urine becomes dark after prolonged standing due to oxidation processes. The urine becomes dark brown to brown black, which may be seen on clothing where the condition exists. It is seen frequently in small children. The addition of 10% sodium hydroxide results in a brownish black ring at the surface which gradually penetrates downward. Ferric chloride causes a transitory blue color with each drop, and reduction of silver lactate and of ammoniacal silver nitrate proceeds rapidly at room temperature. With Millon reagent a lemon yellow precipitate occurs. With heat this precipitate becomes red. The ammoniacal silver nitrate solution results in the production of a black color when added to alkaptonuric urine.

Millon reagent: Metallic mercury, 1 part; fuming nitric acid, specific gravity, 1.420, 2 parts. Dilute with an equal part of water and after a few hours decant.

Ammoniacal silver solution: Silver nitrate, 26 gm. dissolved in 200 ml. water. Add ammonia. A brown precipitate will form; add more ammonia until this precipitate is redissolved. Make the volume up to 1 L. with distilled water.

This condition is evidently an anomaly of protein metabolism and is probably inherited. It appears to be a rare recessive character in the mendelian sense. Its mode of incidence is remarkably similar to that of albinism, but its presence is not as strikingly apparent as is that condition. This error in metabolism is present from birth and persists throughout life.

TESTS FOR SUGAR IN URINE

The many tests available for the detection of sugar in urine utilize several different principles. Monosaccharides will reduce certain metals in alkaline solution with the aid of heat which is the basis of the Trommer, Benedict, and Fehling tests. Glucose, being fermentable, will produce carbon dioxide in the presence of yeast which has been used not only as a method of identification but also for quantitation. Physical properties, such as the rotation of polarized light, have also been used but are not particularly suitable for the routine laboratory. Caramelization, the principle of the Somogyi assay, offers a simple method of obtaining quantitative results.

Differentiation of sugars found in urine

1. Fermentation is a quality of glucose and fructose.

2. Typical crystalline form of osazone of glucose or fructose differentiates these sugars from lactose.

3. To distinguish between glucose and fructose, the Seliwanoff test (p. 1815) is used, or a polariscopic examination may be made.

4. The presence of pentoses is indicated by a positive reaction with Bial reagent (p. 1815).

QUALITATIVE TESTS FOR GLUCOSE IN URINE* (REDUCTION TESTS)

Benedict method

Reagents:

1. Benedict qualitative solution.
    (a) Dissolve 17.3 gm. copper sulfate, c.p., in 100 ml. distilled water with heat in a small Pyrex beaker.
    (b) In a 1000 ml. Pyrex beaker dissolve 173 gm. sodium citrate, c.p., and 100 gm. anhydrous sodium carbonate, c.p. (if crystalline sodium carbonate is used, 200 gm. are substituted for the 100 gm. of the anhydrous), in about 600 ml. distilled water, with heat.
    (c) Filter solution (b). Add the copper sulfate solution slowly and with constant stirring to the mixture of sodium citrate and sodium carbonate, until all has been added. Rinse the residue in the small beaker into the solution, using a few ml. of distilled water. Cool and dilute to 1000 ml. with distilled water.

Procedure:

1. Place 5 ml. Benedict qualitative solution in a test tube.
2. Add 8-10 drops urine and mix.
3. Place the test tube in a cup containing equal parts boiling water and calcium chloride.
4. Allow the contents of the test tube to boil for 2 min.
5. Let stand until cool.

If glucose is present in the urine, there will be a brick red precipitate. If glucose is present in a very large amount, the entire contents will assume a brick red color. Very small quantities of sugar, as low as 0.015-0.020%, will yield a precipitate. Phosphates yield a white precipitate. The degree of reaction is judged by the amount of precipitate.

Benedict solution, although less susceptible than Fehling solution to reduction by other urinary constituents such as uric acid or creatinine, is however promptly reduced by alkaptonic acids or the conjugated glycuronic acids or by an excess of preservatives such as chloroform, chloral (trichloracetaldehyde), and formaldehyde. When the urine of a patient shows reduction in the first examination, use further tests to determine whether the substance is sugar and the kind of sugar.

Streptomycin in concentration as low as 200 μg/ml. reduces alkaline copper reagents such as Benedict solution. With drug levels greater than 700 μg/ml. it is possible that the reaction obtained with the Benedict reagent may be interpreted incorrectly as a positive test for glycosuria.

Unless the urine contains enough reducing substance to reduce the Benedict solution in the heating time specified, it is not clinically significant. Remove the protein from the urine by acidifying with acetic acid, boiling, and filtering.

Fehling method

Reagents:

1. Fehling solution I.
    (a) Dissolve 34.639 gm. crystallized copper sulfate, c.p., in about 300 ml. distilled water with gentle heat.
    (b) Dilute to 500 ml. with distilled water.
2. Fehling solution II.
    (a) Dissolve 173 gm. sodium potassium tartrate (Rochelle salt) and 100 gm. potassium hydroxide, c.p., in about 300 ml. distilled water.
    (b) Dilute to 500 ml. with distilled water.

Procedure:

1. Mix equal parts of solutions I and II in a test tube.
2. Dilute this with 2 or 3 times the amount of water and boil for a few seconds.

If the mixture remains clear after boiling, the solutions may be used. Continue the boiling, adding the urine to be tested drop by drop. If sugar is present, a yellow precipitate will form. If sugar is present in large amount, the first few drops will yield a precipitate. If the quantity of sugar is small, urine is added until an equal volume has been used. If no precipitate forms, the specimen contains no sugar.

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*"In two- to four-plus specimens a yellow film usually adheres to the inside of the tube and defies removal by soap, brush, or cleaner. It will disappear as if by magic if you drop a pinch of dry bicarbonate of soda into the wet tube, moisten the test tube brush and swish it around in the tube, add a little water, and swish it several times more, rinse, and dry. . . ." Medical Economics: Cleaning of test tubes used in sugar analysis.

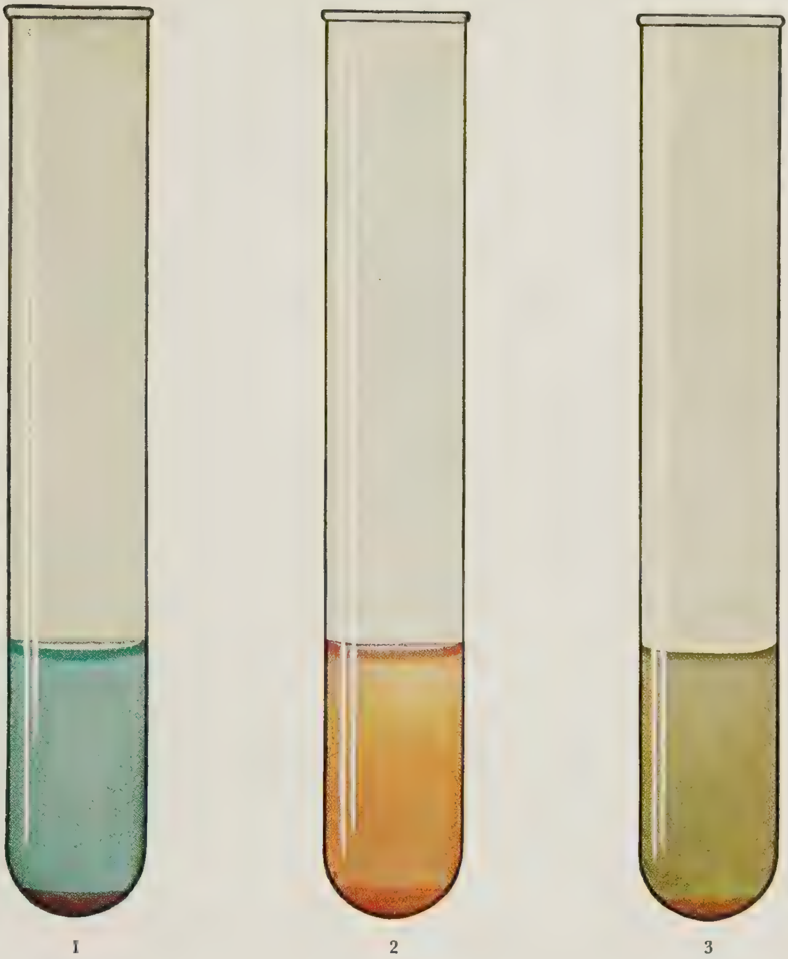

Fig. 615. Benedict test for sugar. **1,** Green—showing only a trace of sugar. **2,** Red—showing a large amount of sugar. **3,** Yellow—showing a small amount of sugar. (From Gradwohl, R. B. H., and Blaivas, A. J.: The newer methods of blood and urine chemistry, St. Louis, The C. V. Mosby Co.)

Nylander method

Reagent:

1. Dissolve 5 gm. Rochelle salts (sodium potassium tartrate) in 100 ml. 10% solution of sodium or potassium hydroxide.
2. Warm and add 2 gm. bismuth subnitrate.
3. Cool and filter through filter paper.

Keep in a dark bottle.

Procedure:

1. Place 5 ml. urine in a test tube.
2. Add 0.5 ml. Nylander reagent.
3. Heat for 3-5 min.
4. Allow to stand for a few moments before reading.

A black color indicates the presence of sugar. A brown color is indicative of a trace of sugar. If the solution turns black after cooling, the reaction is due to substances other than sugar. Phosphates yield a white precipitate. If albumin is present in large amount, remove it by boiling and filtration, since it gives the same reaction as sugar.

Moore-Heller method

1. Place in a test tube 2 parts urine and 1 part 10% potassium hydroxide solution.
2. Boil the upper portion for 2 or 3 min.
3. If phosphates are precipitated in large amount, remove by filtration.

If sugar is present the following reactions take place:

1% or less of sugar yields a canary yellow color
1 to 2% gives a wine yellow color
2 to 3% gives a sherry color
3 to 4% gives a rum color
Above 4% gives a dark brown or black color

Phenylhydrazine test for sugar

1. Place 10 ml. urine in a test tube.
2. Add 0.4 gm. phenylhydrazine hydrochloride and 0.8 gm. sodium acetate.

3. Place the test tube in a water bath and boil for ½-1 hr.
4. Allow it to cool.

Within ½ hr. typical crystals of phenylglucosazone separate when sugar is present, which appear microscopically as fine bright yellow needles arranged in bundles, sheaves, or rosettes.

QUANTITATIVE ESTIMATION OF GLUCOSE IN URINE

Somogyi method

Somogyi[4] has approached the question of carbohydrate utilization or tolerance of diabetic patients along the line suggested by Naunyn and his school at the turn of the century. It is a method of preparing daily balance sheets of carbohydrate consumption and loss of glucose in the urine, the balance representing the amount of carbohydrate utilized in 24 hr. In cases in which insulin therapy is applied, Somogyi advises estimating the total glucose output for 24 hr. periods, running separate quantitative determinations on every sample of urine collected between insulin injections. The information thus obtained proved to be the best criterion for the adequacy of the insulin dosage. One of the advantages of this procedure is that it can be pursued for any length of time without hardship to the patient, whereas gathering of the equivalent information by serial blood sugar determinations is scarcely tolerable for days and nights in a row.

The basis of this method is the well-known change of color which takes place when sugars are heated in alkaline solution; the shade which develops in the reaction varies from pale yellow to dark amber, depending upon the concentration of sugar. Sodium carbonate is the alkali which satisfies the requirements of this test. Glucose quantities varying from 1-30 mg. in 5-6 ml. reaction mixture give maximum color intensities when the concentration of the carbonate is maintained within the range of 8-15%. The maximum color appears after heating alkaline glucose solutions in a boiling water bath for 8 min.; prolongation of the heating beyond 10 min. causes a gradual fading of the color. Boiling over an open flame for 1½-2 min. produces the same colors as heating in the water bath for 7-10 min.

Equipment:

1. Test tubes of 14 mm. inside diameter. All test tubes employed must be of identical diameter. A simple way to select uniform test tubes is to measure accurately 10 or 15 ml. portions of water into a series of tubes and to retain for use only those in which the water level is of the same height.
2. Pipets for measuring 0.5 and 5 ml. portions of fluid.

Reagents:

1. 10% anhydrous sodium carbonate.
   (a) Dissolve 10 gm. anhydrous sodium carbonate in about 50 ml. distilled water and dilute to 100 ml. with distilled water.

Procedure:

1. Introduce into test tubes 0.5 ml. portions of the urines to be analyzed; then add to every test tube 5 ml. sodium carbonate reagent.
2. Mix the contents, place the test tubes in a rack, and heat, immersed in boiling water, for 8 min. Racks accommodating 1-2 doz. (or more) test tubes are serviceable.
3. After 8 min. heating read the results by matching the colors against the standards in the comparator block.*

If the color of the unknown does not closely match any one of the standards but lies between 2 of them, estimate the intermediate value by interpolation. If, for example, the color shade of an unknown is halfway between the 3 and 4% standards, its sugar content is 3.5%; if it is between 3 and 4% but distinctly nearer the 3% standard, then it contains 3.2-3.3% of glucose, etc.

A source of error because of fading of the standards has been eliminated by Somogyi by devising permanent standards. The stock solution for permanent standards is a 0.01N iodine solution in absolute alcohol from which a series of solutions is prepared by dilution with absolute alcohol. The normality of the several diluted solutions is given in Table 358. An 0.008N alcoholic iodine solution is of the same color, shade, and intensity as the 6% glucose standard and thus takes the place of it; a 0.0058N iodine solution is a substitute for a 5% glucose standard, and so on, as given in Table 358.

Table 358. *Concentrations of alcoholic iodine solutions used as permanent standards*

Normality of iodine solutions	*Corresponding percentual glucose concentrations*
0.0004	0.5
0.0009	1.0
0.0018	2.0
0.0028	3.0
0.0043	4.0
0.0058	5.0
0.0080	6.0

Approximately 5 ml. portions of these iodine solutions are introduced into Pyrex test tubes of 14 mm. inside bore, and the tubes are then sealed. In this condition the standards remain unchanged without requiring any special precautions, as, for example, protection from light or changes in room temperatures. The

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*Sets of permanent standards with a matching block were placed on the market by Aloe Scientific, St. Louis, Mo., and are available through laboratory supply houses.

Table 359. *Comparison of results of urinary sugar determinations by Somogyi method with true sugar values estimated by a copper-iodometric method*

	Gm. sugar/100 ml. urine	
No.	*Copper-iodometric method (p. 84)*	*Somogyi method*
1	0.21	0
2	0.42	0.3
3	0.31	0.5
4	0.41	0.3
5	1.2	1.2
6	1.3	1.3
7	2.1	2.0
8	2.6	2.5
9	3.0	3.0
10	3.9	4.0
11	5.0	5.0
12	5.0	5.0

permanence can be readily ascertained by comparison with standards freshly prepared with pure glucose solutions. The earliest permanent standards in Somogyi's laboratory after 2 yr. showed no deterioration.

In Table 359 one can see the results of the Somogyi method with true sugar values estimated by a copper-iodometric method.

This method is as rapid and simple as a properly executed qualitative test for urinary sugar, particularly when one is to carry out considerable numbers of determinations. When only occasional single determinations are to be made, however, preliminary heating of a water bath to boiling and heating of the reaction mixture for 8 min. may involve a waste of time. This difficulty can be overcome by the use of an open flame. Analysis is carried out as follows.

Procedure:

1. Measure into a test tube 0.5 ml. urine and 5 ml. carbonate reagent, drop in 2 small glass beads or quartz pebbles and a drop of paraffin oil (or a corresponding bit of paraffin wax).
2. Heat to boiling over an open flame and, turning the flame low, keep the liquid boiling **very gently** for 1½-2 min.

The color develops to the same shade as after heating for 8 min. in a boiling water bath and can be matched against the permanent standards.

A distinct advantage of the carbonate reaction is that it is more specific for sugars than are copper reagents. This quality comes mainly into play when dealing with concentrated urines, so frequent in hot summer weather, which may reduce appreciable amounts of copper without containing any abnormal amounts of glucose; with Somogyi's method such urines give negative results.

Somogyi further states that if the urine is abnormally dark colored or contains blood or bile, it must be decolorized preliminary to the analysis. To this end add about 2-3 gm. (roughly ½ tsp.) Lloyd reagent* to about 10 ml. urine in a test tube or a small flask, agitate the mixture gently for about 1 min. (or longer), and then filter through a dry filter paper. The nearly colorless filtrate is ready for analysis.

Note: Instead of preparing the permanent standards or purchasing a matching block, it is practical to run standard sugar solutions and to read them in a colorimeter, thereby establishing a standard curve. This would eliminate dependence upon artificial standards as well as permit closer approximations of those specimens that fall in between the standards.

Benedict method

Reagents:

1. Benedict volumetric solutions.
   - (a) Dissolve 18 gm. crystalline copper sulfate, c.p., in 100 ml. distilled water with heat in a small Pyrex beaker.
   - (b) In a 1000 ml. Pyrex beaker dissolve 100 gm. anhydrous sodium carbonate, c.p. (or 200 gm. of crystalline sodium carbonate), 200 gm. sodium or potassium citrate, c.p., and 125 gm. potassium sulfocyanate, c.p., in 600 ml. distilled water with heat.
   - (c) Filter. Add the copper sulfate slowly and with constant stirring to the above mixture. Rinse the residue of copper sulfate in the beaker into the solution with a few ml. distilled water. Transfer to a 1000 ml. volumetric flask, rinsing the beaker with a few ml. distilled water and adding the rinse water to the solution in the flask. Add 5 ml. 5% solution of potassium ferrocyanide. Cool and dilute to 1000 ml. with distilled water. Mix thoroughly; 25 ml. this reagent are reduced by 50 mg. glucose, 52 mg. fructose, 54 mg. galactose, or 67 mg. lactose.
2. To adjust Benedict volumetric solution:
   - (a) 1% glucose solution. Dissolve exactly 1 gm. glucose in distilled water in a 100 ml. volumetric flask and dilute to 100 ml. with distilled water. This solution is not stable.
   - (b) Place in a casserole 25 ml. newly made Benedict volumetric solution.
   - (c) Add a few gm. of sodium carbonate and a pinch of powdered pumice.
   - (d) Titrate as follows: Fill a buret with 1% glucose; record the reading on the buret; boil the contents of the casserole.
   - (e) Add 1% glucose drop by drop to the cas-

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*A hydrated aluminum silicate, used extensively in urine analysis for clarification and also for adsorption of certain alkaloids; obtainable from Hartman-Leddon Co., Philadelphia, Pa.

serole, boiling constantly, until the copper sulfate is reduced to a gray color. Record the reading on the buret. Subtract the first reading from the second.

If more than 5 ml. of a 1% solution of glucose is used, the Benedict volumetric solution is too strong. Adjust as shown in the following example:

6 ml. of a 1% glucose solution were used.
6 ml. contain 60 mg. glucose.
60 : 50 :: x : 25
50x = 1500
x = 30 (vol. to which each 25 ml. solution must be diluted).
30 − 25 = 5 ml. water must be added to each 25 ml. Benedict volumetric solution. There are 975 ml. Benedict volumetric solution on hand.
975 ÷ 25 = 39
39 × 5 = 195 ml. distilled water to be added to a vol. 975 ml.

If the solution is too weak, adjust as shown in the following example:

Second reading 23.0
First reading 18.3
4.7

4.7 ml. 1% glucose were used to reduce 25 ml. Benedict volumetric solution.
4.7 ml. 1% glucose contain 47 mg. glucose.
47 : 50 : : x : 450
50x = 21,150
x = 423 mg. copper sulfate present in each 25 ml. Benedict volumetric solution. Each 25 ml. should contain 450 mg. copper sulfate. This solution lacks 27 mg. copper sulfate for each 25 ml. Benedict volumetric solution.
Vol. 975 ml.
975 ÷ 25 = 39
39 × 27 mg. = 1053 mg. or 1.053 gm. copper sulfate to be added to 975 ml. Benedict volumetric solution.

Procedure:

1. Place the urine in a graduated buret.
2. Place in a casserole of at least 200 ml. capacity 25 ml. Benedict volumetric solution, 5-10 gm. sodium carbonate, and a pinch of powdered pumice.
3. Heat the mixture in the casserole to boiling.
4. Add the urine from the buret drop by drop and very slowly, stirring constantly and boiling the mixture in the casserole throughout the entire procedure. Continue, adding the urine slowly until the last trace of the blue color disappears. This is the end point.
5. Read from the buret the number of ml. of urine used in the reaction.
6. Calculate the quantity of sugar present as follows: 25 ml. Benedict volumetric solution are reduced by 50 mg. glucose. The number of ml. urine used in the titration, therefore, contains 50 mg. glucose. Divide 50 by the number of ml. urine used in the titration, to obtain the number of mg. in 1 ml. urine. Multiply by 100 to obtain the number of mg. glucose in 100 ml. urine. Divide this figure by 1000 to obtain the number of gm. glucose in 100 ml. urine or the per cent of sugar present in the specimen.

Example:

2.5 ml. urine were used in the titration of the specimen.
50 divided by 2.5 is 20.
1 ml. urine contains, therefore, 20 mg. glucose.
100 ml. contain 100 × 20 mg. or 2000 mg. (2 gm.) sugar. The percentage of sugar present is therefore 2.

Fehling method

1. 10 ml. Fehling solution are reduced by 0.05 gm. sugar. Dilute 1 ml. Fehling solution with 4 ml. distilled water in a test tube and heat to boiling.
2. Add urine from a 1 ml. graduated pipet a drop at a time until all the blue color has been removed from Fehling solution. The number of ml. urine used in bringing about this reaction contained 0.005 gm. glucose.

If 0.1 ml. of urine were used in the reduction of Fehling solution, then 0.1 ml. urine contains 0.005 gm. sugar.

1 ml. contains 0.05 gm. sugar.

100 ml. urine contain 5 gm. The percentage of sugar in the urine is therefore 5.

Robert fermentation test

1. Place 4 oz. urine in each of 2 bottles: the first, a bottle of 12 oz. capacity; the second, of 4 oz. capacity.
2. Add a piece of fresh yeast* the size of a walnut to the urine in the 12 oz. bottle and cork; nick the cork for the escape of gas evolved by the fermentation of the sugar. Cork the smaller bottle tightly. Keep both bottles at room temperature.

The fermentation of the sugar will be completed within 24 hr. Take the specific gravity of urine in both bottles. The difference of the specific gravities indicates the number of grains of sugar per fluid ounce. Example:

If the specific gravity of the unfermented urine is 1.035 and of the fermented urine is 1.020, the urine contains 15 grains sugar/fl. oz. or 3%.

This method is not very accurate.

Einhorn fermentation saccharometer

An Einhorn saccharometer is a fermentation tube on a stand with graduations on the arm. It is necessary to have 2 such tubes and 1 test tube for a test.

1. Place 10 ml. urine in a test tube and add 1 gm. fresh yeast.* Shake thoroughly. Pour into the saccharometer, tilting it back until the fluid runs up into the arm, expelling all the air.
2. At the same time fill a saccharometer with normal urine.
3. Allow both to stand at room temperature for 24 hr.

The tube containing the normal urine should show no gas. A small bubble of gas may be disregarded. If the normal urine reacts correctly, the test may be read. Read from the arm of the fermentation tube the amount of sugar present. If the urine contains more than 1% of sugar, it must be diluted with distilled water before testing.

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*Use brewer's or compressed yeast.

Urine of a specific gravity of 1.018-1.020 is diluted twice. Urine of a specific gravity of 1.021-1.028 is diluted 5 times. Urine of a specific gravity of 1.029-1.038 is diluted 10 times.

Millard-Smith micromodification

1. Place exactly 1 ml. Benedict volumetric reagent in a test tube.
2. Add 0.2-0.7 gm. anhydrous sodium carbonate and a pinch of powdered pumice.
3. Draw the urine into a Mohr pipet up to the 100 mark.
4. Heat the Benedict solution to boiling.
5. Add the urine from the Mohr pipet until reduction of the copper sulfate is complete, determined by the disappearance of the blue color. Urines containing more than 3% sugar should be diluted before titration.

Calculate the quantity of sugar present.

One ml. Benedict volumetric reagent is reduced by 2 mg. glucose. If 2 ml. urine were used, then 2 ml. urine contained 2 mg. glucose. One ml. urine contained 1 mg. glucose; 100 ml. urine contained 100 mg., or 0.1 gm. glucose, or 0.1%.

FRUCTOSE IN URINE

Seliwanoff reaction

1. To 10 ml. urine in a test tube add a small amount of resorcin and 2 ml. diluted hydrochloric acid and mix. Heat.

If fructose is present, the solution turns red and precipitates a dark sediment. The sediment is soluble in alcohol, giving a bright red color.

LACTOSE IN URINE

Rubner test

1. To 10 ml. urine in a test tube add 3 gm. lead acetate.
2. Filter off the precipitate and heat the filtrate for a few minutes. A yellowish brown color appears. At this point add ammonium hydroxide and continue heating.

If lactose is present, a brick red color appears in the solution, with a cherry red or copper-colored precipitate. The supernatant fluid becomes colorless after the formation of the precipitate.

Lactose may be found in the urine of women during lactation and in patients who have been on an exclusive milk diet for a long time.

MALTOSE OR ISOMALTOSE IN URINE

Maltose or isomaltose reduces copper solutions but not so strongly as glucose. Ten ml. Fehling solution are reduced by 0.0807 gm. maltose.

CANE SUGAR (SACCHAROSE) IN URINE

Pure saccharose (sucrose) does not reduce Fehling solution.

The finding of saccharose in the urine is of no clinical importance.

PENTOSES IN URINE

The more important pentoses found in urine are rhamnose, arabinose, and xylose. The pentoses reduce copper solutions, the reduction taking place more slowly than with other carbohydrates.

The presence of pentose may be only a temporary or alimentary disturbance due to the ingestion of large amounts of pentose-rich fruits, but it may be pathologic, especially in diabetes. The pentose which is most frequently present in the chronic form is arabinose.

Ten ml. Fehling solution are reduced by 0.0542 gm. pentose.

Fermentation test is negative.

Nylander solution yields a gray precipitate.

Bial orcinol test for pentoses

Bial reagent:

Hydrochloric acid, 30% . .	500 ml.
Orcinol	1 gm.
Ferric chloride solution, 10%	25 drops

Procedure:

1. Boil 5 ml. Bial reagent in a test tube.
2. Remove from the flame and add urine drop by drop until not more than 1 ml. urine has been used. A green color appears almost immediately if pentoses are present.

PROTEIN IN URINE

SIGNIFICANCE OF ALBUMINURIA

Tests for albumin in urine depend upon the visual demonstration of the precipitated protein. The question of what is or what is not pathologic has not entirely been settled. The problem as to which is the best test for protein is also the subject of much debate. The sulfosalicylic acid test is 16 times more sensitive than the Heller test when applied to dilute protein solutions, and when applied to urine the sensitiveness is 3 times as much.

Folin[5] evidently realized the danger of introducing too sensitive a test for routine work and advised a method of using sulfosalicylic acid which is 10-15 times less sensitive than the maximum which can be obtained.

An important question is the significance of small amounts of albumin in urine.

According to life insurance companies, albuminuria in amounts up to 0.03% does not prevent a person under 35 yr. of age and with a negative previous history and otherwise normal findings from being accepted as a standard risk. When, however, individuals with a minute trace of albumin give a history of previous renal disease, then, of course, this finding becomes of great significance.

Insurance authorities divide albumin groups dependent upon the amount of albumin found in various specimens. Daly further divides them into 2 other classes: **intermittent,** in which the albumin is found in ⅔ or less of the specimens examined, and **constant,** in which albumin is found more often. An estimate of the Medico-Actuarial Committee, published in 1938, states that in the class of the **intermittent small amounts,** there were 51,807 exposed to risk, 298 actual deaths against the expected death total of 232, or a mortality ratio of 128% of the expected; in the class of **intermittent moderate amount,** 5881 with 23 actual deaths against an expected death total of 17 deaths, or a mortality ratio of 132%. According to figures of insurance authorities, the mortality is fairly constant in the **intermittent** group, but this does not hold when the albumin is **constant.** In the **moderate amount** of **constant** albuminuria group there were 86 actual deaths against 52 expected, with a mortality ratio of 165%, and in the **large amount** category there were 50 actual deaths against an expected 17 with a mortality ratio of 289%. Expressed in other words, the **constancy** of albuminuria has a definite primary influence on mortality, the **amounts** only a secondary influence. However, the mortality ratio was higher at the older than at the younger ages of entry and increased as the duration of the policies advanced, according to Daly's figures. He states further that the concept of renal disease has changed. Formerly, albuminuria and hypertension were considered definite evidence of Bright's disease; now it is recognized that the hypertension usually comes first and the prime effect is on the cardiovascular system and only secondarily on the kidney. This is reflected in the mortality statistics of insured lives.

The presence of albumin in the urine does not necessarily mean disease of the kidney, for it may at times reflect the reaction to substances, toxic and otherwise, that originate within the body. Physiologic factors may cause transient albuminuria, such as orthostatic and lordotic albuminuria, which will be explained later. In the insurance world albuminuria is but a symptom; albuminuria is considered only from a statistical standpoint. Statistically, one can say that the constancy of albuminuria has a definite primary influence on mortality, the amounts only a secondary influence. However, as just stated, the mortality ratio, according to Daly, was higher at the older than at the younger ages of entry and increased as the duration of the policies advanced. The death rates from nephritis and heart impairment were 5 and 2½ times the normal, respectively.

Albuminuria may be classified clinically as follows:

1. **Febrile albuminuria,** which usually disappears with the fever.
2. **Toxic albuminuria,** which may be either chemical or bacterial: chemical poisoning by phenol, phosphorus, lead, mercury, arsenic, etc.; bacterial toxic albuminuria as is seen in bacterial diseases such as diphtheria, scarlet fever, meningitis, typhoid fever, etc.
3. **Albuminuria due to cardiovascular disease.**
4. **Nephrotic albuminuria;** for example, following leukemia, purpura, syphilis, diabetes, etc.
5. **Obstructive albuminuria** caused by ureteral obstruction from stricture, calculus, or pressure of tumors.
6. **Nephritic albuminuria** (will be fully discussed later; pp. 1817 and 1904).
7. **Neural albuminuria,** such as is seen in certain diseases of the central nervous system; such as epilepsy, brain tumor, cortical injuries, etc.
8. **Alimentary albuminuria,** which occurs in certain individuals from undue ingestion of protein material.
9. **Accidental albuminuria,** in which the albumin comes from other material containing albumin mixed with the urine; such as pus, blood, seminal fluid, etc.
10. **Physiologic albuminuria,** which occurs from violent physical exercise.
11. **Postural albuminuria** (described below).
12. **Benign albuminuria,** which occurs in childhood and adolescence, occurring also in cycles; hence frequently called "cyclic" albuminuria.

Albuminuria is frequently found in young men without any other indications of pathology. Statistical studies in young men with albuminuria disclosed very little difference eventually between the normal subjects, and those with albuminuria. In other words, transitory albumin is likely to be found in young men. College students have been examined, for instance, and have been found to have definite albuminuria in a certain percentage of certain groups. Examination from 5-10 yr. later revealed that in most cases the albuminuria had disappeared.

The past history of these young people must be taken into account in estimating the significance of albuminuria. Those with frequent colds or disturbances in the nose and throat were frequently shown to have albuminuria.

Many different names have been given to this transitory albuminuria of supposedly healthy people, such as orthostatic, postural, lordotic, juvenile, pubertile, cyclic, transitory, etc.

In relation to the so-called postural type of albuminuria, albumin frequently appears in the urine of young men only when they are in an erect posture. It was noted that most of these men with postural or orthostatic albuminuria showed in the standing position a well-marked lordosis, the deepest part of which was at the level of the first and second lumbar vertebrae. This disappeared when the patient reclined. Jehle proved that the production of a lordosis in the erect position accounted for the orthostatic nature of the albuminuria. If a person remained on his knees, a position in which lordosis is very marked, the albuminuria was very pronounced. If the patient, while standing, put one foot on a stool, thus eliminating the lordosis, the albuminuria disappeared. It seems undeniable that the cause of albuminuria in many young people is lumbar lordosis.

The albuminuria which most concerns us as an important diagnostic factor is that seen usually in nephrosis or nephritis. In diseases of the kidney there is an alteration in the secretory function to such an extent that leakage of protein occurs. The urinary protein in renal disease consists of serum albumin and globulin, which are eliminated from the blood plasma. Occasionally in renal disease a cloud forms after the addition of a few drops of 5% acetic acid to the cold urine. Proteins giving this reaction are known as the acetic acid body which does not coagulate on heating. Theoretically it is the result of the presence in the urine of serum albumin and chondroitin-sulfuric acid, which precipitates protein at a faintly acid reaction. In most forms of renal disease, albumin is far more abundant than globulin in the urine.

The amount of protein in albuminous urine varies greatly. The most copious albuminuria occurs in degenerative processes in the renal epithelium; especially great quantities of protein are found in the urine in syphilitic and other chronic nephroses. As much as 5% protein may be found in syphilitic nephrosis.

Regarding the transit of albumin from blood to urine, it seems that urinary proteins have different isoelectric points from the corresponding serum proteins; therefore these must undergo some modification in their transit through the kidney or in the urine.

Some have claimed that proteins are derived entirely from disintegration of the renal cells, but this is questionable. Everything points to the fact that the amount of protein derived from the tubular epithelium is evidently small in comparison with that coming from the blood.

The manner in which protein is excreted in the glomerular portion of the kidney has been the subject of much study. The increased permeability of the kidney to albumin is in line with the fact that the smallest molecular parts of plasma proteins alone pass into the urine. The serum albumins are of smaller molecular weight than the serum globulins, and albuminous urines almost always contain a much higher ratio of albumin to globulin than does the blood. We do not know, of course, what particular influence produces the increased permeability in the glomerular portion.

QUALITATIVE TESTS FOR ALBUMIN IN URINE

Heat test with acetic acid

1. Fill an ordinary test tube ¼ or ⅕ full of of urine.
2. Boil thoroughly.
3. Add 2 or 3 drops of a mixture of equal parts of glacial acetic acid and water.

If albumin is present, the urine becomes cloudy; the greater the cloudiness, the larger the amount of albumin.

The following reactions are possible:

1. If the urine is transparent and remains so, it is normal.

2. If the urine becomes cloudy after boiling but clears after the addition of acetic acid, phosphates are present. If the urine effervesces upon the addition of the acid, either calcium carbonate or ammonium carbonate is present.

3. If the urine becomes cloudy after boiling and remains cloudy or increases in cloudiness, albumin is present. If albumin is present in large quantities, flakes will form and precipitate. Slight cloudiness may be detected by comparison of the test with a test tube of the untested urine. If the 2 test tubes are held against a black background, the faintest cloudiness may be observed.

4. If the urine is cloudy, clears upon boiling, and remains clear after the addition of the acid, urates are present, especially sodium urate.

5. If the urine is cloudy and the cloudiness disappears upon warming but reappears and becomes more pronounced upon boiling with the addition of the acid, urates are present in excess.

6. If the urine is cloudy and remains unchanged by boiling and the addition of the acetic acid, microorganisms are present.

Purdy modification of the heat test with acetic acid

This test is made to eliminate the possibility of nucleoalbumin and mucin.

1. Filter the urine and fill a test tube about ⅔ full of the filtered urine.
2. Add ⅙ its volume of saturated aqueous solution of sodium chloride.
3. Add 5-10 drops 50% acetic acid.
4. Gently heat the upper inch or so of the mixture for ½ min.

If albumin is present, it appears as a cloud in the upper, boiled portion of the test tube. It must be examined in a good light. Even the minutest traces of albumin may be detected by this test.

Heller test

1. Place a small quantity of pure nitric acid in a test tube.
2. Stratify a small amount of urine on the nitric acid.

A white ring at the point of contact indicates the presence of albumin. Urates appear in a ring above the point of contact. Uric acid and urea are recognized by their crystalline nature. Mucin is precipitated but is dissolved by the nitric acid at the point of contact. Nucleoalbumin and albumoses are precipitated. Nucleoalbumin gives a fainter ring than albumin and is above the point of contact. Proteoses disappear upon heating and reappear upon cooling. They are also soluble in picric acid.

Potassium ferrocyanide test

1. Place 10 ml. filtered urine in a test tube and add 5 drops glacial acetic acid. A precipitate should be removed by filtration.
2. Add a few drops of a 5-10% solution potassium ferrocyanide. If a small amount of albumin is present, a faint cloudiness at once appears. If a large amount of albumin is present, a flocculence occurs. Proteose gives a cloudiness, which disappears upon heating.

Spiegler test—modified by Jolles

Reagent:

Mercuric chloride	10 gm.
Succinic acid	20 gm.
Sodium chloride	20 gm.
Distilled water	500 ml.

Procedure:

1. Filter the urine.
2. Place 5 ml. filtered urine in a test tube and add 1 ml. 30% acetic acid solution.
3. Stratify with 4 ml. Spiegler reagent.

If albumin is present, a white zone will be formed at the point of contact.

Sulfosalicylic acid test

1. Place 4 or 5 ml. urine in a test tube and add 2 or 3 drops 20% aqueous solution of sulfosalicylic acid and mix thoroughly.

Opalescence occurs if small amounts of albumin are present. If large quantities of albumin are present, a pronounced turbidity is noted. A heavy precipitate may accompany the turbidity. Proteose is precipitated but disappears on heating, reappearing on cooling.

Biuret reaction

1. Place the urine in a test tube and treat first with 10% sodium or potassium hydroxide solution and then with a 10% cupric sulfate solution. The copper sulfate is added drop by drop.

If serum-albumin and globulin alone are present, the mixture becomes pure violet. If albuminoses and peptone alone are present, it turns rose. If several of the albumins are present together, the urine assumes tints between violet and rose.

Do not add too much copper sulfate because its color will obscure a faint reaction.

Picric acid test

1. Filter the urine.
2. Place 5 ml. urine in a test tube.
3. Add an equal amount of saturated solution of picric acid.

If albumin is present, a precipitate forms, varying from a light cloud to a heavy flocculence, according to the quantity of albumin.

Proteoses are dissolved.

Roberts test

Reagent:

Saturated aqueous solution magnesium sulfate (made by adding 76.9 gm. magnesium sulfate to 100 ml. distilled

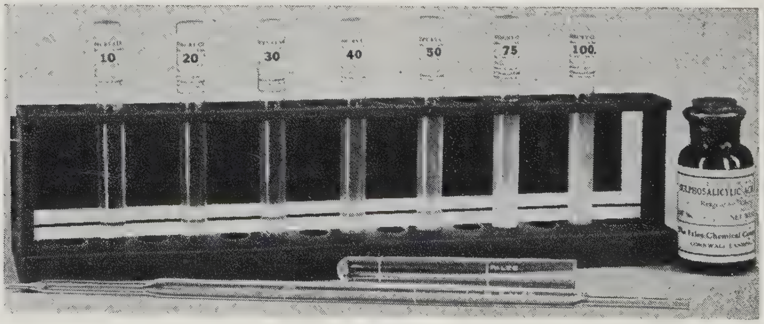

Fig. 616. Kingsbury-Clark albumin kit. (Courtesy Fales Chemical Co., Cornwall Landing, N. Y.)

water) . . . . 5 parts
Concentrated nitric
acid, c.p. . . . . 1 part

Procedure:

1. If the urine is cloudy, filter.
2. Place 1 ml. Robert reagent in a test tube.
3. Stratify 1 ml. urine on the reagent.

A white ring at the point of contact indicates albumin; the larger the ring, the greater is the quantity of albumin. Proteose also precipitates with this reagent but is soluble in picric acid. We highly recommend this test.

QUANTITATIVE TESTS FOR ALBUMIN IN URINE

Kingsbury-Clark method

The advantages of this test are as follows:

1. Uric acid and resin do not react with sulfosalicylic acid.
2. The mucinlike substances are not appreciably affected.
3. The definite amount of albumin is ascertained.

Procedure:

1. Pipet 2.5 ml. centrifuged urine into a test tube graduated at 10 ml. and add 3% sulfosalicylic acid (30 gm. dissolved and diluted to 1000 ml. with distilled water) to the 10 ml. mark.
2. Invert the tube to mix, allow to stand 10 min., and compare the turbidity with the permanent turbidity standards.
3. Record the value of the standard most closely matched, as the albumin content of the urine.

Seven standards are used, equivalent to 10, 20, 30, 40, 50, 75, and 100 mg. albumin/100 ml. urine. In case the specimen contains more than 100 mg. albumin it may be quantitatively diluted and redetermined. By multiplying the value found by the number of times the specimen has been diluted, the desired result is obtained.

A detailed description of the method will be found in the original paper by Kingsbury et al.[6]

A value of 50 mg. albumin/100 ml. urine obtained by this method corresponds to what is usually designated as a "trace" when the Heller or heat and acetic acid test is used.

Although the albumin standards are referred to as being permanent, this is not strictly true. After about 9 mo. the lighter standards tend to become more so and thus yield results which are somewhat too high. The entire set of standards should be replaced every 9 mo.

The principle of the method is that clarified urine is treated with sulfosalicylic acid, and the degree of turbidity is measured by comparison with standard turbidities.

The 7 standards, representing 10, 20, 30, 40, 50, 75, and 100 mg. albumin/100 ml. urine, are corresponding degrees of turbidity uniformly suspended in gelatin medium. Carefully matched test tubes are used to contain the standard turbidity masses to compare perfectly with the empty calibrated specimen test tube in which the test is conducted.

The urine should be filtered or centrifuged. Comparison of the test with the various standards is made with a special comparison rack, and the albumin content is recorded as the value of the standard it most closely matches in turbidity. The comparison rack is best located so that uniform north light enters through the rear, striking through the standards and viewed by the observer at an eye elevation of about 12 in. or so.

It goes without saying that the calibrated tubes in which the test is carried out should be of the same dimensions as the standards.* The standards, when not in use, should be kept in the daylight rather than in a dark cupboard to prevent taking on a dark green tinge. Exposure to undue heat will prove harmful to the standards.

Esbach method

Esbach reagent:

1. Dissolve 1 gm. picric acid, c.p., and 2 gm. citric acid, c.p., in distilled water.
2. Dilute to 100 ml. with distilled water.

Procedure:

1. Render the urine acid by adding a few drops diluted hydrochloric acid, testing with blue litmus paper after the addition of each small quantity; then filter. If the quantity of albumin is very great, dilute accurately with distilled water before making the test.
2. Fill the Esbach tube to mark U with urine.
3. Add Esbach reagent until the liquid stands at mark R. Take care to prevent the formation of bubbles. Close with a rubber stopper.
4. Mix by inverting several times and allow to stand in an upright position for 24 hr. Read from the tube the height of the coagulum, which will be the number of gm. albumin in 1000 ml. urine. To obtain the percentage, divide this by 10. If the urine has been diluted, multiply the final result by the dilution.

Purdy method

1. Place 10 ml. urine in a graduated 15 ml. centrifuge tube.
2. Add 2 ml. 50% acetic acid.
3. Add 3 ml. 10% potassium ferrocyanide.
4. Mix and allow to stand for 10 min.
5. Centrifuge at 1500 r.p.m. for 3 min.
6. Read off the volume of precipitate on the test tube.

Use Table 360 for calculations. The equivalent on Table 360 gives the number of gm. of dry albumin in 100 ml. urine. If the volume of the 24 hr. specimen is 1500 ml. and the precipitate in the centrifuge tube stood at 1.2, then the number of gm. of dry albumin in the 24 hr. specimen is as follows:

Equivalent to 1.2 in Table 360 is 0.25 gm./100 ml. In 1500 ml. urine there are 15 times 0.25 gm. or 3.75 gm.

If the amount of albumin is very large, dilute the urine accurately, and after calculation multiply the final result by the dilution.

Kwilecki modification of Esbach method

1. Place the urine in the Esbach tube to mark U.
2. Add 10 drops 10% solution of ferric chloride and mix.
3. Fill the tube to mark R with Esbach reagent and mix.

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*These tubes, with the standards, can be obtained from the Fales Chemical Co., Cornwall Landing, N. Y.

Table 360. *Showing the relation between volume of precipitate after centrifugation and gravimetric percentage of albumin in the Purdy test*

Vol. precipitate (ml.)	*Gm. % dry albumin*	*Vol. precipitate (ml.)*	*Gm. % dry albumin*	*Vol. precipitate (ml.)*	*Gm. % dry albumin*
0.05	0.010	1.4	0.292	3.2	0.667
0.10	0.021	1.5	0.313	3.3	0.687
0.15	0.031	1.6	0.333	3.4	0.708
0.20	0.042	1.7	0.354	3.5	0.729
0.25	0.052	1.8	0.375	3.6	0.750
0.30	0.063	1.9	0.396	3.7	0.771
0.35	0.073	2.0	0.417	3.8	0.792
0.40	0.083	2.1	0.438	3.9	0.813
0.45	0.094	2.2	0.458	4.0	0.833
0.50	0.104	2.3	0.479	4.1	0.854
0.60	0.125	2.4	0.500	4.2	0.875
0.70	0.146	2.5	0.521	4.3	0.896
0.80	0.167	2.6	0.542	4.4	0.917
0.90	0.187	2.7	0.563	4.5	0.938
1.0	0.208	2.8	0.583	4.6	0.958
1.1	0.229	2.9	0.604	4.7	0.979
1.2	0.250	3.0	0.625	4.8	1.000
1.3	0.271	3.1	0.646		

4. Place in a water bath at 72° C. for a few minutes.

The result may be read at once.

Tsuchiya modification of Esbach method

Tsuchiya reagent:

Crystalline phosphotungstic acid	1.5 gm.
Concentrated hydrochloric acid	5 ml.
Alcohol, 95%	93.5 ml.

Use this reagent instead of the Esbach reagent in the Esbach tube and read after 24 hr.

LaMotte outfit for determination of albumin and sugar in urine

The LaMotte albumin and sugar outfit is very useful for simple, accurate, quantitative tests for albumin and sugar in urine. The Kingsbury-Clark sulfosalicylic acid method is used for the albumin determination, and the Benedict picrate method for sugar.

Urine is treated with sulfosalicylic acid solution, and the resulting turbidity is compared with prepared turbidity standards. The percentage of albumin in the urine is obtained from the value on the label of the tube with which a match is obtained.

The sugar determination involves treating the urine with picric acid in alkaline solution, and development of the color by heating in a water bath. Per cent of sugar is obtained by comparison with permanent color standards.

PROTEOSES IN URINE

Both deuteroproteose and heteroproteose are found in urine in pathologic conditions. They may be divided into 2 groups—**primary** and **secondary**. Primary proteoses are precipitated upon half saturation with ammonium sulfate and secondary proteoses upon complete saturation.

1. Acidify the urine with acetic acid and filter off any precipitate of nucleoprotein which may form.
2. Boil for several minutes.
3. Filter while hot to remove the albumin and globulin.

Test the filtrate by overlaying a saturated solution of trichloracetic acid. A white ring at the point of contact indicates the presence of proteoses. If the test is positive, the primary and secondary proteoses may be separated by half and complete saturation with ammonium sulfate.

BENCE JONES PROTEIN

Significance of Bence Jones protein: The presence of Bence Jones protein in the urine of a patient suspected of having multiple myeloma is supportive evidence of this disease but is not diagnostic. This protein is found in 35-65% of the cases of proved multiple myeloma.

The first reference in the literature to Bence Jones protein appeared in *Philosophical Transactions of the Royal Society* for 1848 in a paper presented by H. Bence Jones on "A new substance occurring in the urine of a patient with mollities ossium." His paper stated, "On the first of November, 1845, I received from Dr. Watson the following note, with a test tube containing a thick, yellow semi-solid substance. The tube contained urine of a very high specific gravity; when boiled it became highly opaque; on the addition of nitric acid it effervesces, assumes a reddish hue, becomes quite clear, but as it cools, assumes the consistence and appearance which you see: heat liquefies it. 'What is it?' "

At the present time, Bence Jones proteins should be regarded not as chemical entities but rather as a class of proteins whose physical state is altered in vitro by temperature changes. That there are multiple Bence Jones proteins is further supported by differences in (1) solubility, (2) electrophoretic mobility, (3) sedimentation velocity, and (4) serologic properties of constituent protein substances. Some investigators conjecture that a patient with multiple myeloma excretes this protein, that it is the result of tumor cell metabolites. Ellinger demonstrated these proteins in the blood, also in ascitic fluid. Yet it has been shown that in the presence of Bence Jones proteinuria, heat-coagulable serum proteins may be absent.

Martin and Mathieson[7] suggest the name pyroglobulinemia to indicate the presence of heat-coagulable globulin in the blood. This is in contrast to the cryoglobulins which are serum protein gels that appear on exposure to cold and redissolve on warming. Cold-coagulable globulins have been described in association with multiple myeloma and also in association with other conditions. The same applies to heat-coagulable globulins. The cases reported to date have, in the main, concerned victims of multiple myeloma, but Hammarsten and associates referred in their work to sera which are sensitive to heating as being found occasionally in chronic infections and in instances of hyperglobulinemia in otherwise clinically healthy persons. Martin and Mathieson reported 1 case of pyroglobulinemia associated with multiple myeloma, but in 4 cases this association did not obtain. In one of the latter cases the patient had lymphosarcoma. There is at times no correlation between the occurrence of pyroglobulinemia and Bence Jones proteinuria.

The evidence at hand favors the conclusion that Bence Jones protein is an extremely small globulin, or group of small globulins, with a molecular weight of approximately 40,000 to 50,000. Its small size may well account for its frequent occurrence in the urine as contrasted with the rarity with which it has been noted in the blood. It has been reported in the following conditions other than multiple myeloma: metastatic carcinoma of the bone, multiple sarcoma of the bone, senile osteomalacia, fibrocystic disease, comminuted fracture, tumor of the jaws, lymphocytic leukemia, myelocytic leukemia, polycythemia, adenocarcinoma of the stomach, carcinoma of the kidney, inactive pulmonary tuberculosis, and prostatitis.

The following is a scheme for processing urine suspected of containing Bence Jones protein, especially in the presence of other protein.[8]

Principle: Bence Jones protein has been described as a urinary protein which is soluble at room temperature and near the boiling point but which coagulates at temperatures beginning at 45°-60° C., reaching maximum insolubility at 55°-70° C.

Reagents:

1. Nitric acid, concentrated.
2. Hydrochloric acid, concentrated.
3. Acetic acid, 25%.
4. Sulfosalicylic acid, 50%.

Procedure:

1. Add 2 or 3 ml. of concentrated HNO_3 to a small test tube.
2. Gently overlay with a few ml. filtered urine.
3. After approximately 3 min. evaluate the precipitate according to the scheme recommended by Jacobson and Milner[8] (Table 361).

Table 361. *Degree of opacity related to protein concentration*

Degree of opacity	*Gm. %*	*Appearance*
1+	0.010-0.018	Not visible against a light background but barely visible when viewed obliquely against a dark background
2+	0.018-0.040	Visible obliquely but not from above
3+	0.040-0.40	Visible but not opaque from above
4+	Over 0.40	Opaque from above

4. If the above test is negative, Bence Jones protein is presumed to be absent. If positive, proceed with step 5.
5. Perform a ring test using concentrated HCl as in steps 1-3.
6. If negative, Bence Jones protein is absent, and the test is discontinued. If positive, proceed with step 7.
7. If the HNO_3 test is greater than 3+, the urine must be diluted with a **protein-free urine** to a concentration approximately 3+.
8. Adjust about 15 ml. urine (original or diluted) to pH 5 with indicator paper, using 25% acetic acid.
9. If a precipitate forms within a period of 5 min., filter and discard the residue (nucleoprotein).
10. Pipet 5 ml. of the urine or filtrate from step 9 into a test tube, place in a water bath, warm slowly to 70° C., and maintain this temperature for 15 min.
11. If no precipitate appears, Bence Jones protein is absent. If positive, proceed to step 12.
12. Raise the temperature to the boiling point for 10 min. If the precipitate dissolves upon shaking, Bence Jones protein may be present.
13. If the precipitate does not dissolve, add 0.5

ml. concentrated HNO_3 and maintain temperature of water bath at boiling point for 10 min. If the precipitate dissolves, Bence Jones protein may be present.

14. If the precipitate does not dissolve, repeat step 10, using another aliquot of the original or diluted urine.
15. Centrifuge and discard supernatant liquid unless the determination of the presence of proteoses or heat-coagulable proteins is desired.
16. Suspend the residue from step 15 in 5 ml. fresh, normal, protein-free urine.
17. Add 0.5 ml. concentrated HNO_3 and heat to boiling point for 10 min. as in step 13. If the precipitate dissolves, Bence Jones protein is present.
18. If the precipitate does not dissolve (pseudo Bence Jones protein), filter while **hot** into a test tube placed in the boiling water bath. Do not allow cooling to occur during this step.
19. Allow the filtrate to cool at room temperature. If the filtrate remains clear, Bence Jones protein is absent.
20. If a precipitate forms, add 0.5 ml. 50% sulfosalicylic acid. If the precipitate dissolves, Bence Jones protein is absent.

Notes: The following comments are numbered to conform to the step numbers in the procedure.

1. The nitric acid ring test is always positive when proteins are present in sufficient amounts.
5. The hydrochloric acid ring test is useful as an additional screening test.
7. It is necessary to adjust the protein concentration to 0.4 gm.% or less to maintain proper response to temperature. Normal urine must be used as the diluent since Bence Jones protein dissolves at boiling temperatures only in the presence of the proper amounts of salt and urea.
8. The proper adjustment of the pH is necessary to obtain maximal precipitation of Bence Jones protein at 70° C.
9. Nucleoprotein, which precipitates at pH 5 at room temperature, is a source of difficulty in that it obscures the presence of Bence Jones protein.
10. The temperature should be controlled to 70° C. with frequent stirring and a thermometer in the solution. Rapid temperature rise should be avoided since actual precipitation begins at about 45°-60°.
11. The appearance of a precipitate at 70° C. does not necessarily prove the presence of Bence Jones protein.
12. Albumin often obscures the re-solution of Bence Jones protein. Therefore, the fact that no apparent re-solution is noticed should not be taken to indicate the absence of Bence Jones protein.
13. It is important to add the proper amount of HNO_3 and to heat for the proper time to avoid hydrolysis and solution of non-Bence Jones protein.
18. Hot filtration is necessary to prevent precipitation and loss of Bence Jones protein. The steam from the water bath serves to heat the funnel used to separate the non-Bence Jones proteins.
19. Rapid cooling should be avoided to prevent the precipitation of urates which may be present. Bence Jones protein which begins to appear as a precipitate at about 70° C. does not redissolve upon further cooling.
20. Sulfosalicylic acid will dissolve urates and cause further precipitation of protein.

Remarks:

1. Fresh urine specimens should be used to avoid the possible conversion of other proteins to pseudo Bence Jones protein or the decomposition of Bence Jones protein which would change its properties.

2. Two other methods that do not depend upon the peculiar solubility-temperature relationship of Bence Jones protein have appeared in the literature.[8a, 8b] The first method utilizes differences in solubility in phosphate solution, whereas the other uses differential solubility in *p*-toluene sulfonic acid.

3. Bence Jones protein is considered to be a urinary rather than a serum component because of its rapid excretion, undoubtedly due to its relatively low molecular weight of about 40,000-50,000.

4. Although the presence of Bence Jones protein is considered to be characteristic for multiple myeloma, it is actually found in only about half of the cases.

5. The amount of Bence Jones protein excreted varies considerably, from trace quantities to 20-30 gm./day.

Qualitative test (presumptive)

1. Place 10 ml. urine in a test tube in a water bath with a thermometer and heat very slowly and gently.
2. Observe frequently. Turbidity will begin to occur at about 40° C., and precipitation will take place at about 60° C.
3. Now acidulate very slightly with acetic acid and raise the temperature to the boiling point (100° C.). The precipitate now partly or totally disappears. Filter while still boiling hot.

The precipitate returns as the tube cools. Precipitation at so low a temperature and dissolving at a higher temperature is typical of Bence Jones protein. It is this characteristic that differentiates it qualitatively from all other protein material occurring in urine.

Test the filtrate by overlaying a saturated solution of trichloracetic acid. A white ring at the point of contact indicates the presence of proteoses. If the test is positive, the primary and secondary proteoses may be separated by half and complete saturation with ammonium sulfate.

1. Precipitate the protein with nitric acid. This precipitate should disappear on boiling and reappear upon cooling.

2. Precipitate the protein with alcohol and col-

lect immediately by centrifuging. The precipitate should be soluble in water.

SPECIAL TESTS

Special urinary tests are described in the following discussion: indican, acetone bodies, bile, bile acids, urobilin and urobilinogen, uroresin, urinary phenols, hemoglobin, hematoporphyrin, coproporphyrins and uroporphyrins, screening test for porphobilinogen in the presence of urobilinogen and indole derivatives, drugs and occult blood.

INDICAN IN URINE

Indican is derived from indole, a product of intestinal putrefaction of albuminous substance. The indole is absorbed by the blood and is oxidized in the tissues to indoxyl, which combines with potassium sulfate and is eliminated in the urine. It is present in minute amounts in every normal urine.

Indican is increased following diets rich in protein and in cases of obstruction of the small intestine, also in intestinal indigestion and in diseases of the stomach associated with decreased hydrochloric acid, gastritis, and gastric cancer. It is also increased in diminished bile flow and in cases in which there is decomposition of proteins in other parts of the body, as occurs in peritonitis and empyema, and other pus collections.

QUALITATIVE TESTS FOR INDICAN IN URINE

Obermayer method

Obermayer reagent:

Fuming hydrochloric acid, c.p.	1000 ml.
Ferric chloride, c.p. . . .	3 gm.

Method 1:

1. Precipitate 20 ml. urine with 5-10 ml. 10% lead acetate solution and filter.
2. Add an equal volume of Obermayer reagent to the filtrate. Shake thoroughly.
3. Add 5 ml. chloroform. Shake thoroughly.

A **blue** coloration of the chloroform indicates indican. A **pink** color is due to the ingestion of iodides.

Method 2:

1. To 5 ml. urine in a test tube add 5 ml. Obermayer reagent.
2. Mix and add 15 drops chloroform. Shake and allow the chloroform to settle.

A **blue** colorization of the chloroform indicates the presence of indican. A **pink** color is caused by ingestion of iodides.

Jaffe method

1. To a small amount of urine in a test tube add an equal volume of hydrochloric acid, c.p.
2. Mix and add 10-15 drops chloroform.
3. Add drop by drop a strong fresh solution of calcium hypochlorite, shaking after the addition of each drop. A blue color appears if indican is present.

A 0.5% solution of potassium permanganate may be used in place of the calcium hypochlorite if preferred. Five drops of the potassium permanganate are used with 10 ml. urine.

Jolle method

1. To 10 ml. urine add 2 ml. 20% lead acetate solution, shake, and filter. To the filtrate add 0.5 ml. 10% alcoholic solution of thymol, 10 ml. Obermayer reagent, and 4 ml. chloroform.
2. Mix thoroughly.

The chloroform takes on a pronounced violet color if indican is present.

QUANTITATIVE ESTIMATION OF INDOXYL COMPOUNDS IN URINE

Sharlit method[9]—modified by Meiklejohn-Cohen[10]

Indican, which is indoxyl potassium sulfate, is the most familiar indole derivative in urine. Part of the indole excreted in urine may occur in a form which is responsible for the urorosein reaction (as yet no satisfactory quantitative method has been developed for the latter).

Reagents:

1. Potassium persulfate solution, 1%.
   (a) Dissolve 1 gm. potassium persulfate in enough distilled water to make 100 ml. solution.
2. Thymol 1% in 95% alcohol.
   (a) Dissolve 1 gm. of thymol in enough 95% alcohol to make 100 ml. solution.
3. Trichloracetic acid, 25%, in hydrochloric acid.
   (a) Dissolve 25 gm. trichloracetic acid in enough hydrochloric acid to make 100 ml. solution.
4. Thymol-free alcohol.

Preparation of urine for test:

1. Place 10 ml. unfiltered urine, preserved under toluene, in a 100 ml. volumetric flask. (It is not necessary to use fresh urine. Addition of a few ml. toluene to the urine when first voided is sufficient to maintain the values obtained by this method for at least 24 hr.)
2. Dilute to 100 ml. with distilled water.
3. Measure 5 ml. diluted urine (containing 0.5 ml. undiluted urine) into a 15 ml. graduated centrifuge tube.
4. Add 0.5 ml. 1% potassium persulfate solution, 0.5 ml. 1% alcoholic thymol, and 5 ml. 25% trichloracetic acid in concentrated hydrochloric acid.

Preparation of blank for test: Prepare a blank determination, using urine and reagents in the same quantities as in the test, but substituting **thymol-free alcohol** for the thymol-alcohol reagent.

High blank readings are sometimes obtained with the use of thymol-free alcohol. This is due to nonspecific substances which on oxidation by persulfate form colored products soluble in ethyl trichloracetate. Such substances might include urorosein and perhaps bile products. Preliminary treatment with basic lead acetate, as advocated by some workers, may sometimes result in a significant reduction in the colorimeter reading, due to the removal of such interfering pigments.

Procedure:

1. Mix the solutions well in their various test tubes.
2. Place the tubes in a boiling water bath until the ethyl trichloracetate has filtered out and the aqueous layer is essentially free from pigment. This requires about 20 min.
3. Centrifuge briefly.
4. Remove as much as possible of the supernatant aqueous layer, using a pipet.
5. Take up the trichloracetate layer together with the small volume of aqueous layer remaining in the centrifuge tubes in sufficient glacial acetic acid to bring the volume to the 5 ml. mark.
6. Transfer to special tubes for use in a Klett-Summerson photoelectric colorimeter. Thoroughly mix by pouring back and forth between the centrifuge tube and the colorimeter tube.
7. Read immediately in the photoelectric colorimeter, using a No. 540 filter.

Calculation: Subtract the logarithmic scale reading given by the blank determination from the reading of the unknown. Calculate the μg indican/ml. urine as follows:

$$\text{Colorimeter reading} \times \frac{0.38}{1000} \times \text{vol. urine} = 24$$

hr. excretion of indican (μg)

If the reading on the colorimeter is less than 0.200 or more than 0.400 optical density units, greater accuracy may be achieved by repeating the test, using appropriately greater or smaller amounts of urine.

Meiklejohn and Cohen[10] found that the factor necessary to convert colorimeter readings into terms of the amount of indican present varies somewhat over the range employed, or, in other words, when colorimeter readings are plotted against concentrations of indican, the resulting calibration curve is not absolutely linear, as required to fulfill Beer's law exactly. The differences between the factors for each concentration of indican are so small that for practical purposes one factor may be used throughout. The factor 0.38 is employed to convert colorimeter readings into terms of μg indican/ml. solution. This factor was obtained from the following equation:

$$\text{Factor} = \frac{\text{Indican in } \mu\text{g}}{\text{Colorimeter reading} \times \text{vol. indican sol.}}$$

$$\text{or } \frac{50}{262 \times 0.5} = 0.38.$$

5-Hydroxyindoleacetic acid[11]

Principle: The normal urinary metabolite of serotonin, 5-hydroxyindoleacetic acid, is diazotized and coupled, producing a purple product. In normal quantities the color is too faint to be seen.

Reagents:

1. 1-nitroso-2-naphthol, 0.1% in 95% ethanol.
   (a) Dissolve 0.1 gm. in 100 ml. 95% ethyl alcohol.
2. Nitrous acid.
   (a) Prepare 2.5% sodium nitrite ($NaNO_2$) by dissolving 2.5 gm. salt in 100 ml. water.
   (b) Just before use add 0.2 ml. sodium nitrite to 5 ml. 2N sulfuric acid.
3. Ethylene dichloride or ethyl acetate.

Procedure:

1. Into a test tube pipet 0.2 ml. urine. Always run a normal urine for comparison and to check the reagents.
2. Add 0.5 ml. nitrosonaphthol reagent and mix.
3. Add 0.5 ml. freshly prepared nitrous acid and mix.
4. Let stand at room temperature for 10 min.
5. Add 5 ml. ethylene dichloride or ethyl acetate and shake vigorously. If turbid, centrifuge.

Results: A purple color in the top layer is positive. A 24 hr. specimen containing 40 mg. shows a detectable color. One having 300 mg. is almost black.

ACETONE BODIES IN URINE

Diacetic acid, acetone, and β-oxybutyric acid are called the acetone bodies. Diacetic acid is formed first, and the others are derived from it. The chief source of these acetone bodies lies in abnormal catabolism of fats. For further particulars about these bodies, refer to Chapter 8.

DIACETIC ACID IN URINE

Gerhardt method

The urine must be fresh.

To a few ml. urine in a test tube add 10% ferric chloride solution drop by drop until no further precipitation occurs.

If diacetic acid is present, the urine will turn a Bordeaux-red color. If the diacetic acid is due to drugs, it does not disappear upon heating. True diacetic acid disappears upon heating and does not reappear upon cooling. If the reaction is dubious, acidulate some of the urine with sulfuric acid and shake up with ether. Remove the ether with a pipet and shake up with very dilute ferric chloride solution. If diacetic acid is present, a violet color results, which changes to a Bordeaux red upon further addition of ferric chloride.

Arnold method—modified by Lipliawski

Reagents:

1. Solution I.

Para-aminoacetophenone	1 gm.
Distilled water	100 ml.
Hydrochloric acid, concentrated	2 ml.

2. Solution II.

Potassium nitrite (KNO_2)	1 gm.
Distilled water	100 ml.

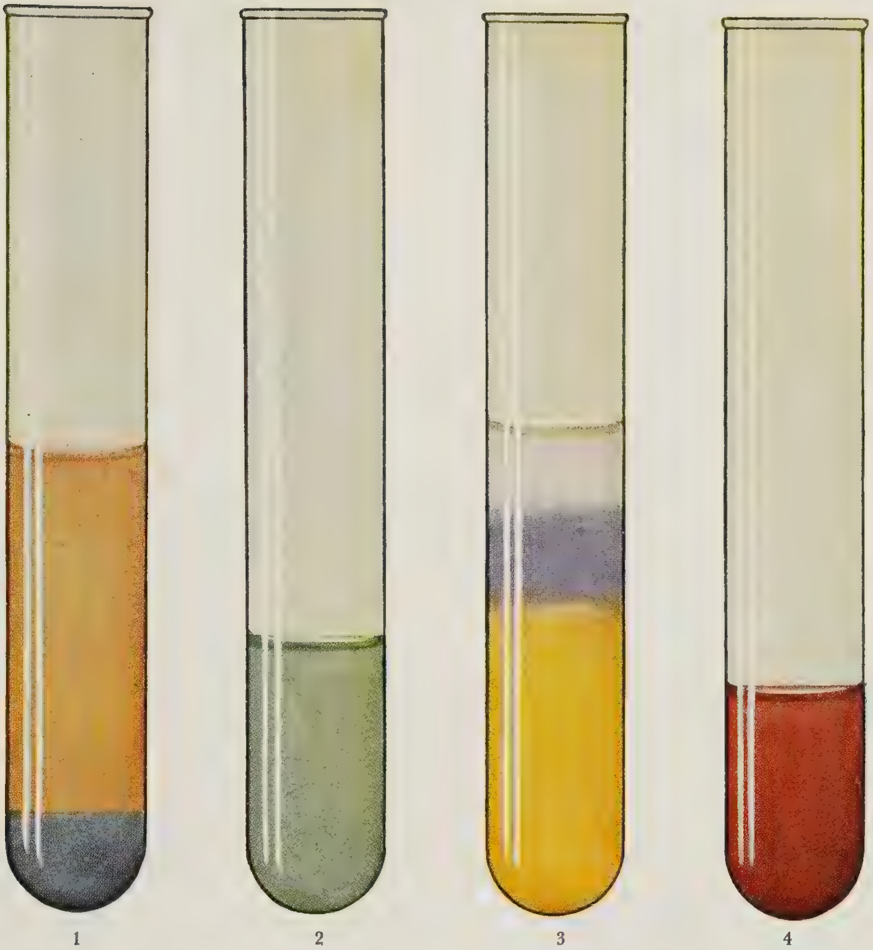

Fig. 617. Urine color reactions. **1,** Indican reaction. **2,** Benzidine test for blood. **3,** Acetone reaction. **4,** Diacetic acid reaction. (From Gradwohl, R. B. H., and Blaivas, A. J.: The newer methods of blood and urine chemistry, St. Louis, The C. V. Mosby Co.)

Procedure:

1. Place 6 ml. solution I in a test tube.
2. Add 3 ml. solution II.
3. Add an equal volume urine and 1 drop concentrated ammonia. Shake the mixture until it assumes a brick-red color.
4. Treat from 10 drops-2 ml. of this mixture (according to probable amount of diacetic acid present) with 15-20 ml. concentrated hydrochloric acid, 3 ml. chloroform, and 2-4 drops of a 10% aqueous solution of ferric chloride. Close the tube with a cork and shake gently for ½-1 min.

If diacetic acid is present, the chloroform becomes tinged with violet. If no diacetic acid is present, the color is yellow or light red.

ACETONE IN URINE

Legal method

1. Dissolve a few crystals of sodium nitroprusside in 2 ml. distilled water in a test tube.
2. To a few ml. urine add enough sodium or potassium hydroxide solution to render it distinctly alkaline.
3. Add a few drops of sodium nitroprusside solution.
4. Add a few drops of concentrated acetic acid.

A purple or violet-red color indicates acetone. Alcohol or acetic aldehyde and diacetic acid also appear red.

Jackson-Taylor modification of Legal test

1. To a few ml. urine add a few drops of a freshly prepared solution of sodium nitroprusside (a few crystals in 2 ml. water).
2. Mix thoroughly and add 1 gm. ammonium sulfate powder.
3. Mix and stratify on this 2 ml. ammonium hydroxide.
4. Allow to stand several minutes.

A purplish red or permanganate-colored ring at the point of contact indicates the presence of acetone.

Lieben iodoform test

1. Add a small amount of phosphoric or hydrochloric acid to 200 or 300 ml. urine, in the proportion of 3 ml. to 100 ml. urine.
2. Distill this mixture.

The tests are performed with the first 10 or 15 ml. distillate. To the distillate add a few drops of strong potassium hydroxide solution and a few drops of iodine and potassium-iodide solution. If acetone is present, a yellow precipitate of iodoform develops at once. This can be easily identified by the characteristic odor. Microscopically, there will be seen thin, yellow hexagonal plates or starlike groups of crystals.

Gunning modification of Lieben test

To prevent confusion with alcohol, Gunning suggests the addition of an excess of alcoholic iodine solution and some ammonia to the distillate or to the urine itself. Iodoform is deposited if acetone is present. The liquid often turns black at first, but as the nitrogen iodide settles and disappears, the yellow iodoform deposit can be recognized.

Do not warm the mixture during the period that it contains nitrogen iodide.

Frommer test

1. To 10 ml. urine in a test tube add 1 gm. potassium hydroxide. Do not wait for a complete solution.
2. Treat the mixture with 10-20 drops of a 10% alcoholic solution of salicylaldehyde.
3. Warm to 70° C.

An intense purplish red ring appears at the point of contact if acetone is present.

BETA-OXYBUTYRIC ACID IN URINE

Hart method

1. To 20 ml. urine add 20 ml. distilled water and a few drops of acetic acid. Boil, reducing the volume to 10 ml. This removes the acetone and diacetic acid.
2. Dilute to 20 ml. with distilled water.
3. Mix and divide the contents equally into 2 portions.
4. To one of the quantities add 1 ml. hydrogen peroxide. Warm gently and allow to cool.

Apply the Legal test for acetone to both tubes (p. 1825). Allow them to stand for a few hours.

If β-oxybutyric acid is present, the tube containing the hydrogen peroxide will show a red ring at the point of contact, whereas no reaction will be detected in the other tube.

BILE IN URINE

These tests depend upon the oxidation of bile pigments by acids with the formation of a series of colored derivatives like biliverdin (green), bilicyanine (blue), and choletelin (yellow). The most important pigment of this group is bilirubin.

Gmelin test

1. Place a small quantity of concentrated nitric acid containing a small amount of yellow nitrous acid in a test tube.
2. Stratify a small amount of urine on the acid.

A band of colored rings at the point of contact of the 2 fluids indicates the presence of bile. These rings are green, blue, violet, red, and yellow. Green is the most pronounced.

Rosenbach test

1. Filter the urine through pure white filter paper.
2. Apply a drop of nitric acid containing yellow nitrous acid to the inside of the filter. A band of colored rings will appear around the nitric acid if bile is present.

Ultzmann test

1. To 10 ml. urine add 3 or 4 ml. 25% potassium hydroxide solution and an excess of pure hydrochloric acid.

If bile is present, the mixture becomes a definite green color.

Maher test[12]

This qualitative test for bile in the urine was developed during the course of determinations of sulfonamide drugs in urine by the Maher-Camp modification of the Marshall method.[13]

1. Place 10 ml. urine in a test tube and acidify by the addition of 1 ml. 20% solution of *p*-toluenesulfonic acid. Ten per cent hydrochloric acid may also be used.
2. Two min. later add 1 ml. 0.1% freshly prepared solution of sodium nitrite.
3. Mix the contents of the tube well.

Development of a green color indicates the presence of bile, presumably by the oxidation of bilirubin to a green derivative. No other substances produce a similar reaction.

BILE ACIDS IN URINE

Hay test

In the Hay test advantage is taken of the fact that bile acids have the property of reducing the surface tension of fluids in which they are contained.

1. Cool the urine by placing it in a refrigerator for several hours.
2. Sprinkle upon the surface a little finely powdered sulfur (flowers of sulfur).

If the sulfur sinks at once, bile acids are present in the amount of 0.01% or more. If the sulfur sinks only after gentle agitation, bile acids are present in 0.0025% or more. If the sulfur remains floating, even after gentle shaking, bile acids are absent.

Oliver test

The Oliver test depends upon the principle that a precipitate is formed of a protein (peptone) and bile acids.

Reagent:

Peptone	8.33 gm.
Salicylic acid	1.12 gm.
Water containing 2 drops acetic acid	1000 ml.

Procedure:

1. Filter a small amount of urine (5 or 10 ml.) until perfectly clear.
2. If the urine is neutral or alkaline, acidify with acetic acid.

3. Dilute with water until the specific gravity is less than 1.008.
4. Place 2 ml. in a test tube.
5. Add 5 ml. reagent.

A positive reaction is indicated by a milky turbidity.

UROBILIN AND UROBILINOGEN IN URINE

It is claimed that urobilin is excreted as the chromogen urobilinogen, which is changed by light into urobilin within a few hours after the urine is voided.

Urobilinogen is increased whenever there is an excess of bilirubin formed through excessive destruction of erythrocytes, especially in pernicious anemia and malaria. When the liver cells fail to function properly, there is an increase, and for this reason the test has become of practical value as a **liver function test.**

A marked decrease or even total absence may occur in obstructive jaundice when the obstruction is complete or nearly complete. If the obstruction is only partial, urobilinogen excretion may be normal or even increased.

UROBILIN IN URINE

Schlessinger test

1. To 10 ml. urine in a test tube add a few drops of Lugol solution to transform the chromogen into the pigment.
2. Add 10 ml. saturated alcoholic solution of zinc acetate. Mix and filter.
3. View the filtrate in sunlight against a dark background or with light concentrated upon it with a lens; a greenish fluorescence indicates the presence of urobilin.

Bile pigment, if present, should be previously removed by adding ⅕ vol. of 10% solution of calcium chloride and filtering.

Ehrlich test

Reagent:

Paradimethylaminobenzaldehyde	10 gm.
Hydrochloric acid, concentrated	75 ml.
Water	75 ml.

Procedure:

1. Qualitative test.
   (a) Place 10 ml. urine in a test tube 15 mm. in diameter. The urine should not be too cold. If very cold, allow it to stand at room temperature or gently heat to between 65° and 80° F.
   (b) Add 1 ml. Ehrlich reagent and mix.
   (c) Allow to stand for 3-5 min.

At the end of 3-5 min. a cherry red color appears if urobilin is present in abnormal amount. A light red and shades of pink appear when it is present in normal quantity. The examination for color should be made by viewing the contents through the mouth of the tube, holding it at a slight angle over white paper.

If no color appears, the tube should be allowed to stand longer, and then if no color appears, it should be heated and again examined before reporting an absence of urobilinogen.

This test is to some extent quantitative. With a little experience one can judge by the color whether there is an increase above normal.

2. A **quantitative test** may be conducted as follows.
   (a) Place 6 test tubes (15 mm. in diameter) in a rack. In them place the following amounts of urine: No. 1: 10 ml. 1:20, No. 2: 10 ml. 1:30, No. 3: 10 ml. 1:40, No. 4: 10 ml. 1:50, No. 5: 10 ml. 1:100, and No. 6: 10 ml. 1:200. The water used for diluting should not be too cold. If tap water is used, it may be necessary to add enough warm water to bring it to about room temperature (between 65° and 85° F.).
   (b) To each tube add 1 ml. Ehrlich reagent and mix.

At the end of 5 min. examine the tubes by viewing through the mouth against a white background. Note the highest dilution which shows the slightest pink color. This is reported as positive 1:50, 1:200, etc.

When possible the readings should be made by daylight, as artificial light has a tendency to intensify the color. Highly concentrated urines may give a yellowish brown discoloration which must be differentiated from the true pink reaction of urobilinogen.

Bile pigment, if present, should be previously removed by adding 1 part of 10% solution of calcium chloride to 4 parts of urine and filtering.

UROBILINOGEN IN URINE

Wallace-Diamond method

Ehrlich aldehyde reagent:

1. Mix 20 ml. concentrated hydrochloric acid with 80 ml. distilled water.
2. Add 2 gm. *p*-dimethylaminobenzaldehyde.
3. Stir until dissolved.

Procedure:

1. Add 1 ml. Ehrlich aldehyde reagent to 10 ml. urine.
2. Warm the test tube.

A rose color appears if urobilinogen is present. The amount may be determined by diluting the specimen and observing the dilution at which the reaction is still present.

Watson quantitative test for urobilinogen in feces and urine[14]

Reagents:

1. Ferrous sulfate solution, 20%.
   (a) Dissolve 20 gm. ferrous sulfate previously ground to a powder in a mortar in 92 ml. distilled water.
2. Sodium hydroxide solution, 10%.
3. Modified Ehrlich reagent.
   (a) Dissolve 0.7 gm. of *p*-diamethylaminobenzaldehyde in 150 ml. concentrated hydrochloric acid and add to 100 ml. distilled water.
4. Saturated aqueous solution sodium acetate.
5. Petroleum benzine (petroleum ether).
6. Glacial acetic acid.

Feces:

1. Collect feces for a 4 day period, mix well, and grind a 10 gm. portion in a mortar with a few ml. distilled water.
2. Dilute to 300 ml. with distilled water or to only 100 ml. if the feces are definitely acholic.
3. Pour into an Erlenmeyer flask containing 100 ml. 20% ferrous sulfate solution.
4. Add with constant mixing, 100 ml. 10% sodium hydroxide solution.
5. Cork and allow to stand 1 hr. If the flask stands overnight, slightly lower values are obtained.*
6. Filter.
7. To 2 or 3 ml. filtrate add an equal amount of modified Ehrlich reagent.
8. Mix and add several ml. saturated aqueous solution of sodium acetate.
9. Depending upon the amount of color, use from 1-50 ml. filtrate in the quantitative procedure. If the color is intense, use 1 ml. or less; if pale red, use 5-10 ml.; if faint, use 25 ml.; if absent, 50 ml. The amount of filtrate chosen should be only enough that the final colored solution does not exceed 100 ml. and that its color is not more than 50% on the standard scale.
10. Place the amount of filtrate decided upon in a 125 ml. pear-shaped separatory funnel. Dilute filtrate to 20 ml. with water if less than this amount of filtrate has been chosen.
11. Cover with 25 ml. purified petroleum benzine (petroleum ether).
12. Strongly acidify with glacial acetic acid.
13. Shake vigorously for several seconds.
14. Separate the aqueous solution of petroleum benzine and wash twice with distilled water.
15. Shake vigorously with 1 or 2 ml. modified Ehrlich reagent.
16. Add at least twice as much of a saturated solution of sodium acetate. The maximum color now appears.
17. Shake vigorously and then separate the colored solution into a 100 ml. graduated cylinder.
18. Again extract the petroleum benzine with Ehrlich reagent. Repeat until the urobilinogen has been entirely removed as evidenced by no further development of color.
19. Follow each extraction by the addition of sodium acetate, shaking, and separation of the aqueous fraction into the cylinder.
20. Make the colored solution up to a volume convenient for calculation and mix.
21. Fill the right-hand tube of the Hellige-Dunning colorimeter† and compare with the standards. Should the intensity of color be greater than 50% of the standard, dilute the specimen further and read again. Comparisons are most accurate between 20 and 50%. The intensity of the color may also be compared with that of standards in a photoelectric colorimeter in the usual manner.

Calculation:

$$\frac{\text{ml. ferrous hydroxide mixture}}{\text{gm. feces}} \times \frac{\text{ml. final colored solution}}{\text{ml. filtrate used}} \times \%\ \text{color standard} \times \frac{\text{weight of 4-day amount feces}}{100} \times \frac{1}{\text{No. days collection of feces}} = \text{mg. urobilinogen/day.}$$

Example:

$$\frac{500}{10} \times \frac{45}{3} \times 0.35 \times 2.8 \times \frac{1}{4} = 183.75 \text{ mg./day.}$$

Standardization of the colorimeter in terms of crystalline mesobilirubinogen (urobilinogen) has been described.[14]

Urine:

1. Reagents are the same as for feces.
2. Collect a 24 hr. sample urine in a dark bottle containing approximately 5 gm. sodium carbonate and 100 ml. purified petroleum benzine.
3. Separate urine from petroleum benzine and measure.
4. Place 50 ml. mixed urine in a 500 ml. Erlenmeyer flask.
5. Add with thorough mixing 25 ml. 20% ferrous sulfate solution and 25 ml. 10% sodium hydroxide.
6. Let stand 1 hr., then make a qualitative determination, and then use this information in selecting the amount of urine to be used in the quantitative procedure.

Proceed as with the filtrate from feces. Compare in the Hellige-Dunning* colorimeter.

Calculation:

$$\frac{100}{50} \times \frac{\text{ml. final colored solution}}{\text{c.c. of filtrate used}} \times \%\ \text{standard} \times \frac{\text{vol. 24 hr. urine}}{100} = \text{mg. urobilinogen in 24 hr.}$$

Example (hepatic dysfunction):

$$2 \times \frac{20}{2} \times 0.18 \times 14 = 50.4 \text{ mg. in 24 hr.}$$

Watson's figures show that the amount of urobilinogen excreted in the urine in 24 hr. by the normal adult varies between 0 and 4 mg., usually 0.5-2 mg. He noted a great drop in the amount of urinary urobilinogen coincident with the reticulocyte response to liver therapy in cases of pernicious anemia, together with considerable decrease in the amount of urobilinogen in the feces. The normal range for the feces is between 40 and 280 mg./day, usually 100-250 mg. His conclusions based upon his exhaustive work regarding urobilinogen in feces were that inanition, inactivity, or a low-grade infection tends to lower the excretion of urobilinogen; fever of any considerable degree probably tends to increase the amount. He noted increases in the excretion of urobilinogen in feces in cases of pernicious anemia, Hodgkin's disease, and leukemia. Low values were encountered in certain cases of secondary hyperchromic anemia and in all the cases of hypochromic anemia investigated. Slight increases were observed in half the cases of polycythemia vera.

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*Slightly lower values are obtained if the mixture is allowed to stand overnight. For this reason the values given subsequently are undoubtedly slightly too low since the mixture was allowed to stand overnight in most of these instances.

†Hynson, Westcott, & Dunning, Inc., Baltimore, Md.

*A permanent glass standard colorimeter for determination of phenolsulfonephthalein in urine made by Hynson, Westcott, & Dunning, Inc., Baltimore.

In another paper in reference to the excretion of urobilinogen in the common forms of jaundice and diseases of the liver in 135 patients examined, Watson found that jaundice due to stone is not accompanied by a considerable increase in the amount of urobilinogen in the urine unless complications are present, such as acute cholecystitis or choleangitis, biliary cirrhosis incident to persistent biliary obstruction, or severe anemia. These complications are accompanied, according to Watson, by a relatively marked increase, the actual amount of urobilinogen appearing in the urine being dependent to some extent on the degree of biliary obstruction and the consequent amount of urobilinogen formed in the bowel and reabsorbed from it.

Diffuse hepatic disease is characterized by a marked increase in urobilinogen in the urine; some of the instances of idiopathic diseases of the liver in the group of patients with cirrhosis were accompanied by evidence of an increased destruction of blood, i.e., an increase in the urobilinogen content of the feces and regenerative anemia, often macrocytic.

Increases in the amount of urinary urobilinogen in hemolytic jaundice could not be correlated with increased destruction of blood, but it occurred when other factors, which are believed to disturb the function of the liver, also were present. Urobilinogen content of the feces was usually increased to a marked degree, in the majority of cases decreasing rapidly after splenectomy.

BILIRUBIN IN URINE

Methylene blue test

In February, 1933, Fellinger and Menkes published their article on "quantitative bilirubin determination in the urine, with methylene blue method."[15] Franke had previously suggested the use of methylene blue for the quantitative determination of bilirubin in the urine by determining the number of drops of methylene blue required to change a positive green color to a definite dark blue color. Twenty drops of a 2% solution of methylene blue represented roughly 1 mg. bilirubin present in the urine. In low concentrations the change from green to blue is definite. With greater concentrations of bilirubin in the urine, the change is not so clear-cut, and the accuracy of the results is impaired by the increased volume of methylene blue in the test.

A standard containing a known amount of bilirubin was later developed. A definite bluish green hue is obtained by mixing a solution of 1 ml. 0.2% methylene blue and 5 ml. alkaline fluid containing 1 mg. bilirubin. This bluish green hue is then matched by preparing a solution of alcoholic methylene blue in a weak potassium dichromate solution which is kept in an airtight container in a dark room. The test is then carried out by adding drop by drop a 0.2% methylene blue solution to 5 ml. of urine until it matches the prepared standard.

Myers[16] in 1945 stated that it is not definitely known whether the methylene blue's changing to green in the presence of bilirubin is the result of a chemical reaction or due to a mixture of pigments. The latter seems to be more probable. The presence of the urobilin pigment normally found in urine will not give a positive test even when the urine is very dark and concentrated. The only known false positive results encountered in these tests have been found in urine containing blood and in several cases in which there was an elevated temperature above 101° F. The possibility that the hemosiderin pigment of the blood may play a vital part in this test is likely. Two drops of blood hemolyzed in 10 ml. water will give a slightly positive test when methylene blue is added, and if the number of drops of blood is increased, the green color is intensified. Istizin is the only drug that will give a false positive reaction.

A test for occult blood must be made on the urine before making the methylene blue test because if occult blood is present a false positive methylene blue test results.

To keep all factors as constant as possible, the size of the tubes, the volume of the test, and the number of drops of methylene blue for each test must never vary. Use tubes 16 × 150 mm.

Procedure: Titrate 5 ml. urine with 0.25% aqueous solution of methylene blue.

Normal urines require less than 4 drops (0.2 ml.) to give a blue color.

Interpretation: In a study by Helfferich the following conclusions were made. In hepatitis the methylene blue test reached a maximum a few days before serum bilirubin; in icterus due to obstruction this test was more sensitive than following serum bilirubins although the results of both ran parallel; in cirrhosis the methylene blue remained elevated even after serum bilirubin had returned to normal; paper chromatography of a mixture of methylene blue and bilirubin showed that the 2 substances did not form a stable complex.

Modification of Harrison test for bilirubin in urine

Bilirubin may appear in the urine in experimentally induced hepatitis before there is any elevation in the total serum bilirubin. Accordingly, there is need for a rapid test of the urine to detect the presence of bilirubin. Harrison[17] suggested a method which has been named for him. In this test barium chloride solution and urine are mixed in equal amounts, precipitate is caught on filter and then is treated with Fouchet reagent which gives a green color if bilirubin is present. Hawkinson et al.[18] described a modification better adapted for usage in large numbers of tests. They stated it is equivalent to the original method of Harrison.

Reagents:

1. Fouchet reagent.
    (a) Dissolve 0.9 gm. ferric chloride in enough 25% trichloracetic acid to make 100 ml. solution.
2. Barium-impregnated strips* or cotton swabs.
    (a) Allow pieces of extra thick and retentive filter paper (Schleich and Schull No. 470) to remain briefly in a saturated aqueous solution of barium chloride. Allow them to dry in the air or preferably in a drying oven, and then cut them into strips 4 in. long × ½ in. wide. Use a single strip for one test, as described below. If a suitable type of thick filter paper is not available, cotton swabs may be used, although the resulting test is not quite as sensitive and clear-cut as with the filter strips.
    (b) Prepare the swabs on ordinary applicator sticks, the cotton having dimensions of about 1½ × ½ in.
    (c) Dip these into the saturated aqueous solution of barium chloride; after drying, they are ready for use.

Procedure:

1. Place one end of a barium chloride-impregnated thick filter strip in the urine sample to be tested, the strip being in an approximately vertical position, at least ½ extending above the surface of the sample.
2. Allow the strip to stand in the urine for 30 sec. up to 2 min., then withdraw it, and place it on a piece of dry paper such as a paper towel or any other absorbent paper. Inspection of the filter strip will usually reveal somewhat more color in that area which corresponded to the surface of the urine.
3. Place 2 or 3 drops of Fouchet reagent directly on this area.

A positive test is denoted by the appearance of a green color, varying in intensity with the amount of bilirubin present. With smaller amounts the color is often detected as a faint green line running across the strip.

When the cotton swab is used, the method is varied slightly.

1. Immerse the swab in the urine to be tested.
2. Express the excess urine from the swab by rolling it against the side of the container.
3. Place 2 or 3 drops of Fouchet reagent in the middle of the swab.

A positive test is denoted as just described. The weaker reactions are less easy to interpret, in that the reaction is over a large area and hence more diffuse and not as clear-cut as the line reaction observed with the filter strip method. Nevertheless the swab method has considerable usefulness when the proper type of filter paper is not available.

In a number of instances a series of dilutions of bilirubin-containing urines has been made to a point where the ordinary Harrison test was either questionable or absent. In each instance the results obtained with the present method were entirely comparable. Thus it is believed that the present filter strip method is at least equivalent to the original Harrison procedure.

It may be noted that barium has a peculiar virtue, possibly of catalytic nature, in this reaction. The test is not as sensitive when calcium chloride is used although calcium salts appear to adsorb bilirubin from the urine just as efficiently as barium.

In connection with this test, Stokes and Osterberg[19] reported a study with three objectives: (1) to determine whether any correlation existed between the degree of hyperbilirubinemia and the positivity of the methylene blue test; (2) to attempt to find, if possible, at what concentration of bilirubin in the urine the result of the test became positive, and (3) to demonstrate the nature of the test, i.e., whether the color which is produced represents a chemical reaction or merely a blend of colors.

Twenty-eight patients who had diseases involving the liver and the common bile duct were studied. In all patients there was hyperbilirubinemia, and in 22 patients jaundice was present. There was not perfect correlation between the degree of hyperbilirubinemia and the positivity of the test.

Spectrotransmittance curves of urines giving positive tests and other solutions used in these experiments were determined by means of a Coleman Junior spectrophotometer, Model 6. Urines giving a positive test had a spectrotransmittance characteristic of bilirubin with maximal absorption between 425 and 450 $m\mu$. Stokes and Osterberg found two absorption bands, one at 425 to 450 and one at 660 $m\mu$, showing that the resulting green color is merely a blend of the original yellow and blue. That there is no new substance formed is evidenced by the lack of any new absorption band.

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*Aloe Scientific, St. Louis, Mo.

Their conclusions were as follows.

1. They did not observe perfect correlation between the concentration of bilirubin in the blood serum and the degree of positivity of the methylene blue test.

2. The methylene blue test performed on urine with artificially produced bilirubinuria first became positive at a concentration of 2 mg. bilirubin/100 ml. urine.

3. Under the conditions of their experiments it would appear that the color which results when methylene blue is added to urine which contains bilirubin is due to a blend of blue and yellow colors rather than to some specific chemical reaction between methylene blue and bilirubin.

UROROSEIN IN URINE

Urorosein does not occur in normal urine but may occur in various diseases, such as pulmonary tuberculosis, typhoid fever, nephritis, and stomach disorders. It is excreted as a chromogen (indoleacetic acid) which is transformed into the pigment upon treatment with a mineral acid.

Test

1. To 10 ml. urine in a test tube add 5 ml. concentrated hydrochloric acid and a few drops of 1% solution of potassium nitrite.
2. Mix thoroughly.

A rose-red color indicates urorosein.

URINARY PHENOLS

The determination of urinary phenols deserves study because Becher[20] reported the occurrence of an increased amount of these substances in patients suffering with pernicious anemia. Volterra[21] corroborated this finding. Because of the difficulties incident to chemical analyses covering these ingredients, attention has been directed to a determination of the urinary phenols. In the determination of isocyclic amino acids it is also necessary to determine their intermediary products, aromatic hydroxy acids. To this end Volterra[22] gives a simple technic for the determination of 3 fractions of aromatic substances in the urine.

Volterra technic

1. Use the Folin and Ciocalteu phenol reagent (p. 46 for formula for this reagent).
2. Dilute this reagent with equal volume of distilled water.
3. To 10 ml. fluid in a test tube add 0.5 ml. dilute phenol reagent and 2 ml. 20% sodium carbonate. Shake. After 20-30 sec. immerse the tube in boiling water for exactly 1 min. and then cool under running water.

This technic is divided into 3 separate determinations: (1) determination of volatile phenols, (2) determination of aromatic hydroxy acids, and (3) determination of residual phenols.

Determination of volatile phenols

1. Use 10 ml. precisely measured urine from 24 hr. specimen.
2. Pour into a 250 ml. distillation flask.
3. Add 150-170 ml. distilled water and some sodium carbonate until solution is alkaline to litmus paper; then distill. Free volatile phenols are carried over in the steam. They are present in small amounts; hence stop when 30-40 ml. have been obtained.
4. After the flask cools add 2-4 ml. dilute sulfuric acid until acid to Congo red paper; start distillation again.
5. After 100 ml. are obtained, place 5 ml. specimen in a small test tube, add 0.25 ml. phenol reagent and 1 ml. sodium carbonate, and heat in a water bath.

If the phenol reaction is still present, continue distillation after adding 100 ml. water. To 10 ml. both distillates add phenol reagent and sodium carbonate and carry out the test as described. Prepare a standard solution from a stock solution of 1 mg. phenol/ml. The best suitable standard is that of 3 mg.% diluted 10 times. This 10 fold dilution has the proper intensity for colorimetric reading.

Calculation:

$$\frac{OD_u}{OD_s} \times \text{mg. in standard} \times \frac{\text{ml. distillate}}{100} \times \frac{\text{Quantity of 24 hr. urine}}{100} = \text{mg. volatile phenols in 24 hr. specimen}$$

Determination of aromatic hydroxy acids

1. Transfer the acid solution in the distillation flask to a 250 ml. separatory funnel, dilute with water to 100 ml. and shake for 3 min. with 30-40 ml. petroleum ether.
2. Draw off petroleum ether and shake twice with ether.
3. Wash ether with water and evaporate on steam bath.
4. Add 10 ml. water before complete drying; then complete evaporation.

5. Use 1 ml. of this solution which shows a yellow color with 9 ml. water. Dilute 2 or 4 times.

Use standard phenol solution.

The result multiplied by 1.5 gives the content of aromatic hydroxy acids expressed as mg. *p*-hydroxyphenylacetic acid.

Determination of residual phenols

1. Dilute the residue from the ether extraction with 200 ml. water.
2. To 10 ml. add 1-1.5 ml. silver lactate solution (3% silver lactate in 3% lactic acid) and 1-2 drops colloidal iron. Shake, bring to 20 ml. with distilled water, and filter after 15 min.
3. To 10 ml. add 1-1.5 ml. saturated solution sodium chloride in 10% hydrochloric acid.
4. Bring to 20 ml. with distilled water and filter.
5. Treat 10 ml. filtrate with Folin-Ciocalteu reagent as just described. The result, taking into account the various dilutions, gives the so-called residual phenols.

Volterra's normals are as follows: 44.4 mg. volatile phenols in 24 hr.; according to this figure on normal subjects the daily elimination of aromatic hydroxy acids averaged 71 mg. There was an average of 336 mg. of residual phenols in the normal subject.

HEMOGLOBIN AND DERIVATIVES IN URINE

Hemoglobinuria or hemoglobin in urine is caused by dissolution of red blood corpuscles. It is found in severe diseases, such as yellow fever, smallpox, and scarlet fever, following poisoning with carbolic acid, sulfuric acid, and naphthol, and after extensive burns. There are also records of cases known as paroxysmal hemoglobinurias. See the Teichmann test, p. 1916.

Heller test

1. Precipitate the phosphates from the urine by adding potassium or sodium hydroxide and heating. As they precipitate, they carry with them the hemoglobin and therefore are blood-red, not white. The coloring matter may be detected microscopically.

Almén guaiac test

1. Make tincture of guaiac by adding 1 part guaiac to 100 parts absolute alcohol.
2. Mix equal parts of tincture of guaiac and old oil of turpentine. This mixture must not have any blue color.
3. Stratify on this mixture a small amount of urine.

If hemoglobin is present, first a bluish green ring and then a light or dark blue ring appear at the point of contact. On shaking, the mixture becomes blue.

Pus gives a blue ring but disappears on boiling.

HEMATOPORPHYRIN

1. To 100 ml. urine add 20 ml. 10% sodium hydroxide.
2. Collect the precipitate by centrifuging or filtering.
3. Wash the precipitate with water and then with alcohol.
4. Add 5 ml. alcohol and 5-10 drops hydrochloric acid, c.p., concentrated, mix thoroughly to dissolve and filter. Filtrate must be absolutely clear.
5. Examine spectroscopically for absorption bands of acid hematoporphyrin.

PORPHYRINURIA

The urine excreted is Burgundy red with a brown tinge, but sometimes it varies from pale pink to almost black. It may be normal yellow when passed but darkens on exposure to light. When the feces contain large amounts of porphyrin, they turn almost black on oxidation.

Acute porphyria is apparently congenital. Patients with this condition show no light sensitivity, and porphobilinogen is excreted in the urine in large amounts. The disease is inherited as a mendelian dominant and is more common in women than in men. They show the following symptoms: abdominal symptoms, with severe, cramplike pains, vomiting, and pronounced constipation. The abdomen is distended, and there may be slight fever and tachycardia. The skin may show a diffuse or spotted pigmentation. The nervous system shows involvement by reason of constant pain in the extremities, accompanied by muscular weakness. There is paralysis of the lower motor neuron type, with flaccidity and loss of tendon reflexes. The symptoms suggest Landry's paralysis. Mental symptoms may suggest hysteria. Some patients show toxic delirium with hallucinations of vision and hearing. The urine is dark but may contain only the colorless porphyrinogen.

Hematoporphyrin is found in urine following the administration of such drugs as quinine, tetranol, and others.

Refer to pp. 98 and 1083 for a further discussion of porphyrins.

TEST FOR PORPHYRINS

Porphyrins may be detected by their fluorescence in ultraviolet light or by means of their absorption spectra.

Reagents:

1. Acetic acid, glacial, c.p.
2. Ethyl alcohol, 95%.
3. Ethyl ether, anhydrous, c.p.
4. Hydrochloric acid, c.p., concentrated.

5. Hydrochloric acid, 5% aqueous solution.
    (a) Add 5 ml. concentrated hydrochloric acid to enough distilled water to make 100 ml. solution.
6. Sodium hydroxide solution, 10%.
    (a) Use 10 gm. sodium hydroxide/100 ml. Keep closed.

Procedure:

1. Place 25 ml. urine in a separatory funnel.
2. Add 10 ml. glacial acetic acid.
3. Extract twice with 50 ml. ether.
4. Combine the extractions and wash with 10 ml. 5% hydrochloric acid.
5. Examine the washings under ultraviolet light.

A red fluorescence indicates coproporphyrin. The urinary residue under ultraviolet ray shows a red fluorescence if urinary porphyrins are present.

Confirmatory test:

1. Add 20 ml. 10% sodium hydroxide to 100 ml. urine.
2. Filter and wash the precipitate with distilled water.
3. To the precipitate on the filter paper add 5 drops concentrated hydrochloric acid.
4. Wash into a test tube with the addition of 5 ml. 95% alcohol.
5. Examine the filtrate spectroscopically for absorption bands of hematoporphyrin.

COPROPORPHYRINS AND UROPORPHYRINS

Method of Ham[23]

Examine the urine in a dark room. Expose to ultraviolet light for fluorescence, which indicates the presence of porphyrin.

Separation of coproporphyrin

1. Acidify 100 ml. urine with 10 ml. glacial acetic acid and allow to stand exposed to the air overnight.
2. Place in a separatory funnel and extract 3 times with ethyl ether. Use 1-2 times the volume of the original mixture for each extraction.
3. Combine the ether extracts and wash once with 50 ml. distilled water.
4. Return the distilled water washing to the original urine sample.
5. Extract the **ether extract** 3 times with 2 ml. aqueous hydrochloric acid, 25% by volume.
6. Cool the hydrochloric acid extracts and examine under ultraviolet light.

Porphyrins

1. Adjust the reaction of the previously extracted urine-acetic acid mixture to pH 3.0 to 3.2 by adding either aqueous hydrochloric acid (1% by volume) or solid sodium acetate.
2. Extract the urine with solid ethyl acetate, using 1 or 2 times the volume of the urine for each extraction.
3. Combine the ethyl acetate extracts and wash once with 50 ml. distilled water and save.
4. Extract the ethyl acetate extract further 3 times with 2 ml. amounts 25% hydrochloric acid.
5. Pool the acid extracts and examine for fluorescence and for the absorption spectra.

SCREENING TEST FOR PORPHOBILINOGEN IN PRESENCE OF UROBILINOGEN AND INDOLE DERIVATIVES

Watson-Schwartz method[24]

Reagents:

1. Ehrlich aldehyde—Watson modification.
    (a) Dissolve 0.7 gm. *p*-dimethylaminobenzaldehyde in a mixture of 150 ml. concentrated hydrochloric acid and 100 ml. distilled water. Store in a dark brown glass bottle.
2. Saturated solution sodium acetate.
    (a) Place approximately 1000 gm. sodium acetate ($3H_2O$) in 1 L. volumetric flask. Add distilled water to the liter mark and heat to about 60° C.
    (b) Allow to cool.
    (c) Mix thoroughly.

Procedure: The urine specimen must be **fresh.**

1. Mix 2.5 ml. fresh urine with 2.5 ml. Ehrlich reagent in a test tube.
2. Add 5 ml. saturated sodium acetate solution.
3. Observe for development of a pink color.
4. Add 3-5 ml. chloroform.
5. Shake thoroughly and observe the color of the chloroform layer.

Interpretation: If a pink to red color develops after the addition of Ehrlich reagent and sodium acetate, it may indicate the presence of both urobilinogen and porphobilinogen.

If after extraction with chloroform, the red color remains in the aqueous portion of the test tube, this indicates the presence of porphobilinogen, which is insoluble in chloroform.

If the red color is taken out of the aqueous layer and transfers itself to the chloroform layer, it indicates the presence of urobilinogen and no porphobilinogen since urobilinogen is soluble in chloroform.

If the color remains in both layers, it indicates the presence of both porphobilinogen and urobilinogen.

DRUGS IN URINE

See the section on Toxicology for a discussion of the examination of urine for drugs.

Certain drugs alter the color of urine.

Dark green: phenol, phenyl salicylate, creosote, and resorcinol

Red: antipyrine and trional

Brown or black: pyrogallol

Yellow: phenacetin and picric acid; santonin changes from bright yellow to scarlet with sodium hydroxide

Iodine: Acidify the urine with hydrochloric acid. Add a few drops starch solution and a few drops calcium chloride. In the presence of iodine a blue color develops.

Phenol: If carbolic acid is present, the urine becomes dark on standing, green-brown to black. In carbolic acid-containing urine, sulfuric acid is found.

Salicylic acids—Salol (phenyl salicylate) and salophen (phenetsal): Addition of iron chloride produces a violet coloration. If there is only a trace of these drugs present, acidifying, shaking with equal parts of ether, and then adding diluted ferric chloride will give the same reaction.

Antipyrine with ferric chloride: This gives a red color.

Phenacetin: To a few ml. urine in a test tube add 2 drops concentrated hydrochloric acid, 2 drops 1% sodium nitrite solution, and a few drops alkaline α-naphthol solution. Now alkalinize. A light red color is produced. This becomes yellow on the addition of hydrochloric acid.

Rhubarb and senna: The presence of rhubarb and senna is detected by alkalinizing the urine, which gives a deep red color.

Phenolphthalein: Phenolphthalein, which is a constituent of some purgatives, appears in the urine and can be discovered by the addition of sodium hydroxide solution, producing a red color.

Pyramidon (aminopyrine): Pyramidon in the urine is detected by the addition of tincture of iodine, producing a yellow ring.

Quinine: Quinine may be detected with the following reagent:

1. Solution 1.

Potassium iodide	10 gm.
Hot distilled water	50 ml.

2. Solution 2.

Mercuric silver iodide . . .	2.7 gm.
Distilled water	150 ml.

Mix solutions 1 and 2 together and add 2.5 gm. acetic acid. The addition of this reagent to an alkaline-free urine in the presence of quinine gives a cloudiness. As albumin will also cloud, it should be eliminated. The cloudiness produced by quinine disappears on heating, whereas the cloudiness produced by albumin remains.

Quinine—a simple field test[25]

Reagent:

Mercuric iodide	3 gm.
Potassium iodide	2 gm.
Glacial acetic acid	20 ml.
Distilled water, sufficient to make	60 ml. of reagent.

The reagent must be kept in an amber bottle.

Procedure: One or 2 ml. urine are used for a test. To a small amount of urine (1 or 2 ml.) add 5-10 drops reagent.

An immediate turbidity which appears on slight agitation is an indication of quinine. This intensifies upon standing. This method will detect as little as 0.001 mg. quinine.

Substances such as atropine, cocaine, strychnine, Atabrine, and albumin interfere with the reaction. The presence of the alkaloids would be rare; if the patient has taken these drugs, this fact would likely be known. Albumin may be more frequently encountered; its presence may give a false positive test. In a suspected false positive test a simple differentiation may be made by heating the tube. The quinine precipitate will dissolve with heat and reappear when cooled. The albumin precipitate intensifies when heated and persists.

This test may be of some value in determining whether the patient has been taking and absorbing quinine. It will detect ingestion of a 5 gr. tablet of quinine up to the thirty-sixth hour.

Sulfonamides

Refer to Chapter 12 for methods of testing for sulfonamide drugs.

Antibiotics

Refer to pp. 610 and 713 for a discussion of antibiotic assays.

Arsphenamine
Dicken modification of Autenrieth test for arsenic

Reagents:

1. Concentrated hydrochloric acid.
2. Sodium nitrite, 0.5%.
    (a) Dissolve 0.5 gm. sodium nitrite in 100 ml. distilled water.
3. Resorcinol, 10%.
    (a) Dissolve 10 gm. resorcinol in 100 ml. distilled water; should be colorless.
4. Sodium carbonate solution, 20%.
    (a) Dissolve 20 gm. anhydrous sodium carbonate in 100 ml. distilled water with heat and filter through filter paper.

Procedure:

1. To 10 ml. urine in a test tube add 3 drops hydrochloric acid. Allow to stand 5 min.
2. Add 10 drops 0.5% sodium nitrite solution. Allow to stand 3 min.
3. In another test tube place 5 ml. 10% resorcinol and 3 ml. 20% sodium carbonate solution.
4. Overlay the resorcinol solution with the diazotized urine.

In the presence of arsphenamine a rose-red ring develops slowly at the point of contact of the 2 liquids. In the presence of atoxyl a yellow ring develops. The lower portions of the test tube are used as controls. Mix the contents thoroughly. The color should remain.

OCCULT BLOOD
Phenolphthalin test[26]
Gettler-Kaye method

Phenolphthalin reagent:

1. Place 200 ml. distilled water, 20 gm. sodium hydroxide, and 1 gm. phenolphthalein in a 500 ml. Erlenmeyer flask.
2. When completely dissolved, add 20 gm. granulated zinc (20-30 mesh).
3. Using a reflux condenser to prevent evaporation, slowly boil the mixture until the red color of the alkaline phenolphthalein disappears, leaving a colorless or faintly yellow solution. This may take 2-3 hr. The phenolphthalein is thereby reduced to phenolphthalin which is colorless in alkaline solution.
4. When cool, place in a brown bottle and stopper with a rubber stopper. Some of the zinc may be added; this will aid in keeping it in the reduced form. The reagent will keep for many months if kept in a cool dark place.

Procedure:

1. Place 1 or 2 ml. portions urine, spinal fluid, thin fecal suspension, or gastric contents in a test tube and boil for about 30 sec. in order to destroy any oxydases that may be present.
2. Cool and add about 5 drops phenolphthalin reagent, followed by about 3 drops 3% hydrogen peroxide.

A pink to red color indicates the presence of blood. The intensity of the color is a rough indication of the quantity of blood present. The color may fade after 3 min.

Tests for blood in feces and in gastric contents should be applied only if the patient has been on a meat-free and fish-free diet for 3-4 days. Bleeding, as seen in hemorrhoids, as well as the admixture of menstrual blood, is to be considered in the interpretation of the results.

The phenolphthalin test is slightly more specific for occult blood than are the guaiac, benzidine, and orthotolidine tests and is especially recommended for medicolegal work.

Comparison of the sensitivity of the 4 tests was found to be as follows:

Guaiac test—1 part in 10,000
Benzidine test—1 part in 1,000,000
Orthotolidine test—1 part in 10,000,000
Phenolphthalin test—1 part in 10,000,000 (if sample is fresh); 1 part in 1,000,000 (if sample is old and decomposed)

Benzidine test

Follow the method given on p. 1916, substituting urine for gastric contents.

Benzidine dihydrochloride test

This test has the advantage over the benzidine test mentioned above in that it is used in aqueous solution, and the solution is stable for at least 3 mo.

Reagents:

1. Benzidine dihydrochloride, 1% aqueous solution.*
    (a) Shake 1 gm. benzidine dihydrochloride and 100 ml. distilled water at intervals for about 2 hr. or until dissolved; filter and keep in a dark amber bottle; do not use heat in preparing the solution.
2. Hydrogen peroxide, 3% solution.
    (a) Dilute 30% hydrogen peroxide, c.p., with distilled water in the proportion of 1 vol. 30% peroxide to 9 vol. water.
3. Sodium acetate, 1% aqueous solution.
    (a) Dissolve 1 gm. sodium acetate crystals (reagent) in enough distilled water to make 100 ml. total solution.

Detection of blood in urine:

1. Place 1 or more ml. urine in a clean test tube and dilute to about 5 ml. with distilled water.
2. Add in the order named: 1 ml. benzidine dihydrochloride solution, 1 ml. hydrogen peroxide solution, and 1 ml. sodium acetate solution.

A blue color develops in the presence of blood. The rapidity with which the color develops depends chiefly on the amount of blood present, 1-10 sec. usually being required.

When the amount of blood is very small, centrifuge the urine, pour off the supernatant fluid, and place the sediment on a glass slide. Add in the order named 1 drop benzidine dihydrochloride solution, 1 drop hydrogen peroxide solution, and 1 drop sodium acetate solution and mix by tilting the slide.

Detection of blood in feces: See Chapter 108.

1. Mix about 1 gm. feces in a test tube with 5 ml. distilled water, bring to a boil over a flame, and filter.
2. Collect the filtrate in a clean test tube and cool.
3. Add in the order named 1 ml. benzidine dihydrochloride solution, 1 ml. hydrogen peroxide solution, and 1 ml. sodium acetate solution.

Color reactions are the same as when urine is used in the test.

Orthotolidine test—Stone-Burke[27]

Orthotolidine was first proposed by Phelps in 1909 as a qualitative test for minute amounts of free chlorine or hypochlorites in water. It is a crystalline basic body of the aromatic series obtained by reduction from orthonitrotoluene, having a melting point of 129° C. and is quite insoluble in distilled water but soluble in acid solutions and in alcohol and ether. Ruttan and Hardisty[28] in 1912 reported the use of orthotolidine as a test for the detection of blood, using a 4% solution in glacial acetic acid with a small amount of Perhydrol* as an oxidizing agent to obtain a blue color in the presence of blood.

Stone and Burke reported that they had found orthotolidine useful in the detection of minute quantities of blood in urinary sediments. They pointed out that red cells in urinary sediments are often crenated or disintegrated in specimens a few hours old and may be confused with leukocytes. Red cells are frequently disintegrated in alkaline urine. The continued presence of red blood cells in urinary sediments has important clinical significance by attracting attention to glomerular inflammatory changes, which are frequently overlooked in the absence of albuminuria or other gross evidences of disturbance. Stone and Burke found that red cells were excreted more or less constantly in patients with evident foci of infection, indicating evidences of glomerular inflammation. It is well known, of course, that a few red cells, leukocytes, or casts, in the absence of other symptoms, may have little significance following unusual exercise or exertion.

Reagents:

1. Orthotolidine, 1%, in chemically pure methyl alcohol.

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*Merck Sharp & Dohme, Division Merck & Co., Inc., West Point, Pa.

*Merck Sharp & Dohme, Division Merck & Co., Inc., West Point, Pa.

  - (a) This dissolves with slight difficulty and keeps at least 10 mo.
2. Acetic acid–hydrogen peroxide mixture.
  - (a) Glacial acetic acid, 1 part, and commercial hydrogen peroxide, 2 parts (keeps 3 or 4 mo., probably longer).

Procedure:

1. Centrifuge 15 ml. urine at 1500 r.p.m. for 5 min.
2. Pour off the supernatant fluid.
3. Prepare a portion of the sediment for microscopic examination in the usual way.
4. To the remaining sediment, add 2 drops orthotolidine solution and 2 or 3 drops acid-peroxide solution.

Undiluted blood serum, 10% sodium hydroxide, strong trisodium phosphate solutions, and probably other strong alkalies will give positive reactions. Pus cells or any of the common organic or inorganic constituents found in the urine do not give positive reactions.

Chapter 100

Rapid tests

SAM FRANKEL

A series of special tablets, sticks, and tapes is available for extremely sensitive and rapid analyses of many constituents in urine and other fluids (Table 362).

Table 362.* *Rapid tests and their uses*

Name	*Test*
Combistix	Protein, glucose, and pH
Uristix	Protein and glucose
Clinitest	Sugar
Clinistix	Glucose
Albustix	Protein
Albutest	Protein
Bumintest	Protein
Ketostix	Acetoacetic acid
Acetest	Acetoacetic acid and acetone
Hematest	Occult blood
Occultest	Occult blood
Hemastix	Hematuria
Ictotest	Bilirubin
Phenistix	Phenylpyruvic acid
Dextrotest	Blood sugar

*Available from Ames Co., Inc., Elkhart, Ind.

Also available is the well-known Tes-Tape* for urinary glucose, the lesser known Sheftel urine sugar test case,* and the Urograph,† a quantitative assay of blood urea nitrogen, described on p. 81.

Space limitations will allow only a brief discussion of these materials,‡ being confined for the most part to principles and procedures. In certain cases pertinent comments concerning special precautions will be included. Certain of the materials will be omitted when there is an overlap.

Combistix

This is a multiple test strip, offering a most convenient method of obtaining rapid results for protein, glucose, and pH.

Protein: "The Combistix colorimetric test for protein is based on the well-known phenomenon of 'protein error of indicators,' the fact that at a fixed pH certain indicators will have one color in the presence of protein and another color in the absence of protein. The citrate buffer contained in the protein test area of Combistix provides a hydrogen ion concentration of approximately pH 3. At this pH the indicator tetrabromphenol blue has a yellow color, whereas at the same pH in the presence of increasing amounts of protein the indicator will change yellowish green to green to blue.

Glucose: "Glucose oxidase, reacting with glucose in the urine, removes 2 hydrogen atoms to form gluconolactone, which is promptly hydrated to gluconic acid. The displaced hydrogen atoms are then combined with atmospheric oxygen to form hydrogen peroxide.

$$\text{Glucose} + O_2 \xrightarrow{\text{Glucose oxidase}} \text{Gluconic acid} + H_2O_2$$

"The hydrogen peroxide in the presence of peroxidase oxidizes a colorless dye, orthotolidine, which in its oxidized state assumes a blue color.

$$H_2O_2 + \text{Orthotolidine} \xrightarrow{\text{Peroxidase}} \text{Oxidized orthotolidine (blue)} + H_2O$$

pH: "It is well established that certain dyes may impart a constant color at a specific pH but may change in shade or color with variations in pH. These dyes usually exhibit this phenomenon within clearly defined limited ranges of pH. The combination of the dyes methyl red and bromthymol blue provide a series of bright orange, yellow, green, and blue colors within the range of pH 5.0 to pH 9.0, the essential range of urinary pH.

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*Eli Lilly & Co., Indianapolis, Ind.

†Warner-Chilcott Laboratories, Morris Plains, N. J.

‡Quoted from Products profiles, Ames Co., Inc., Elkhart, Ind.

"Directions:

"*Note:* The reliability of any test depends upon the care with which it is performed.

"Follow directions meticulously.

"1. Moisten the test end of the strip in urine by dipping and removing immediately. (Prolonged immersion may cause leaching of test chemicals.)

"2. Touch tip of strip against edge of container to remove excess urine. (The avoidance of excess urine on the stick helps to prevent urine containing test material spreading from one area to another.)

"3. Read the glucose (mid) test area at **10** sec. after wetting if the glucose color chart is to be used. (The time of reading the pH and protein portions is not critical.)

"4. Hold test portions close to the appropriate color chart and match or interpolate color reactions with the appropriate color charts.

"Important: Good lighting is essential for accurate color interpretations.

"Interpretation of color reactions:

"*Protein test portion (distal test area):*

"*Negative result*—Color closely matches the 0 or negative color block.

"*Positive reaction*—If protein is present, the yellow tip changes immediately to a yellow-green to green to blue-green to blue color depending on the quantity of protein present. With experience and adherence to directions, one can interpolate Combistix color reactions lying between the color blocks on the chart. Color reactions identical with the trace color block indicate approximately 20 mg. protein/100 ml. urine. It is possible to detect lesser amounts of protein.

"*Glucose test portion (midtest area):*

"*Negative result*—No color change occurs.

"*Positive reaction*—If glucose is present, the red area changes from light to medium to dark shades of purple. A light color usually corresponds to a small amount of sugar (less than 0.5% or between 100 and 500 mg./100 ml.) and a dark color to a larger amount (more than 0.5% or 500 mg./100 ml.). A medium color may be produced by either a large or a small amount of sugar. As little as 0.1% (or 100 mg./100 ml.) of glucose in urine can be detected with Combistix. Enzyme tests for glucose are influenced by pH, temperature, and the presence of antagonistic substances such as ascorbic acid. Therefore, they should be regarded essentially as qualitative tests although in many instances they can indicate the presence of small, moderate, or large amounts of glucose.

"*pH test portion:*

"Color blocks on the color chart connote the pH of urine as follows:

Orange: pH 5
Orange-yellow: pH 6
Yellow-green: pH 7
Dark green: pH 8
Dark blue: pH 9

"Colors lying between these pH values can be interpolated.

"Precautions in using Combistix:

"1. Collect urine specimens in clean containers and protect from contamination.

"2. Test fresh urine specimens. Upon long standing, bacterial action may result in highly alkaline urines nullifying pH determinations and sometimes causing false positive protein reactions. (A sufficiently [highly] buffered urine with a pH of 8.0 or above may produce a protein color reaction in the absence of protein.)

"3. Do not touch the Combistix test areas with the fingers, which may have had prior contact with reactive substances.

"4. Do not use Combistix in presence of fumes of volatile acids or bases.

"5. Do not allow the Combistix strip to remain in the urine specimen. Quick dipping prevents possible loss of test materials.

"6. Remove excess urine from strip after dipping by touching tip to edge of container. This prevents urine [from] flowing chemicals from one test area to another.

"7. Be precise in reading the glucose test portion 10 sec. after dipping if the glucose color chart is to be used. A delay in reading will result in excessive color developing in the test area of the strip.

"8. It is essential that all readings be made by holding the test area close to the corresponding color chart. Never read by laying the strip over the collection bottle and looking down the long axis of the strip to the color chart placed in the background.

"9. Never read color reactions in poor light. Lighting must be bright whether daylight or artificial. Dim light may result in false positives or overly high protein readings. If blue fluorescent lights (nondaylight type) are present in the laboratory or if the walls are blue or green, it should be determined if these factors require compensatory readings, particularly with the protein test portion.

"10. Avoid 'overreading' the reactions of Combistix. The successful use of any colorimetric test is dependent upon the experience and color acuity of the technician. Although controlled studies have shown that both trained and untrained operators obtain reliable results with Combistix, there is a tendency for those first using the colorimetric protein test of Combistix to overread slight changes from the negative which should be disregarded."

Clinitest

"Sodium carbonate and a small amount of the citric acid act as an effervescent couple to facilitate rapid solution of the tablet. Reducing sugars present in the test urine react with the copper sulfate to reduce the cupric ions to cuprous oxide, giving a color change of blue through green to orange, depending upon the amount of reducing substances present. The sodium hydroxide provides the alkaline medium necessary for the reaction. The heat required is provided both by the solution of the sodium hydroxide and by the reaction between sodium hydroxide and citric acid.

$$2\ Cu^{++} + \underset{\text{sugar}}{\text{Reducing}} \xrightarrow[\text{alkali}]{\text{Heat and}} Cu_2O + \underset{\text{sugar}}{\text{Oxidized}}$$

"Directions:

"1. Place 5 drops urine and 10 drops water in test tube.

"2. Add Clinitest reagent tablet. Watch reaction carefully. (If solution passes through orange to some shade of brown, it indicates more than 2% urine-sugar present, which should be recorded without reference to color chart.)

"3. Wait 15 sec. after boiling stops; shake gently and compare with color chart.

"**Interpretation:** The Clinitest color chart has 6 color blocks varying from blue through green to orange representing urine sugar levels of 0.25, 0.5, 0.75, 1, 2%, and over."

Albutest

"This protein test is based on the well-known phenomenon of a 'protein error of indicators' which involves the fact that at a fixed pH certain indicators will have one color in the presence of protein and another color in the absence of protein. The salicylate buffer provides a hydrogen ion concentration of approximately pH 3.0. At this pH bromphenol blue has a yellow color, whereas at this same pH in the presence of increasing amounts of protein the indicator will have a green to blue color.

"When a drop of urine containing protein is placed on the tablet, the protein is adsorbed on the tablet surface. Following addition of 2 drops water, a considerable quantity of the urine is washed away, but protein, indicator, and buffer remain at the surface of the tablet, and the indicator produces a green to blue-green color. With a urine containing no protein, the tablet may show some color change when the drop of urine is added because of the local effect of the pH of the urine, but upon the addition of 2 drops water the yellow color of the indicator is restored.

"Directions:

"1. Place tablet on clean surface (preferably a piece of paper) and put 1 drop urine on tablet.

"2. After urine drop has been absorbed, add 2 drops water and allow penetration before reading.

"3. Compare color on top of tablet with color photograph (color photographs on insert accompanying each package).

"Interpretation:

"Negative—If no protein is present the original color of the tablet will not be materially changed at the completion of the test.

"Positive—If protein is present in the urine sample, a blue-green spot will remain on the tablet surface after the addition of water. The intensity of the blue-green spot is proportional to the amount of protein present in the urine."

Bumintest

"When the Bumintest tablet is placed in water, the sodium bicarbonate and a small part of the sulfosalicylic acid present interact with an effervescence that facilitates rapid solution of the entire tablet. The resulting solution of free sulfosalicylic acid precipitates urine proteins which are estimated quantitatively by the degree of turbidity of the solution.

"**Directions:** The reagent solution is prepared by dissolving 4 Bumintest reagent tablets in 1 oz. (30 ml.) water. This produces a 5% solution sulfosalicylic acid that is ready for use as soon as the effervescence has subsided, and it remains indefinitely stable under ordinary conditions.

"1. Place equal parts of reagent solution and urine—not less than 10 drops each—in small test tube.

"2. Shake test tube gently.

"**Interpretation:** The amount of protein present is estimated by the degree of turbidity (Fig. 618).

"Precautions:

"Stability: Bumintest reagent tablets are effervescent tablets and are somewhat hydroscopic. Exposure to moisture should therefore be avoided. The tablets are stable if kept dry

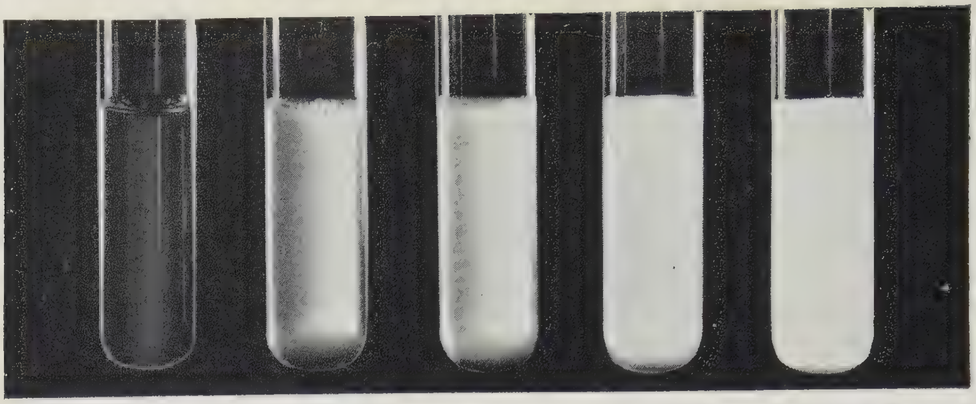

Fig. 618. Turbidity testing of urine for albumin showing negative reaction, trace, small amount, medium amount, and large amount. (Courtesy Ames Co., Inc., Elkhart, Ind.)

and in the original container. It is important that the container be kept securely closed when not in use.

"The solution made from the tablets is stable for at least a month if kept in an amber bottle at room temperature.

"*Urine turbidity:* Urine turbidity may be due to cellular debris, casts, crystals of phosphates, urates, etc. If the urine sample is turbid prior to testing with Bumintest, the sample should be filtered or centrifuged. However, if the turbidity is not great, the test with Bumintest may be done in the usual way, and the reaction is compared with the original solution to determine whether there is an increase in turbidity.

"*Interfering substances:* Metabolites of the following drugs have been found to cause false positive reactions with sulfosalicylic acid turbidity tests: (1) penicillin (massive doses), (2) tolbutamide, and (3) x-ray media (cholecystographic and urographic media).

"When these metabolites are suspected, the specimen should be checked with one of the Ames colorimetric protein tests (Albutest, Albustix, Uristix) which do not give false reactions to these metabolites.

"Occasionally tap water contains sufficient iron to produce a pink color when Bumintest reagent tablets are dissolved in it. In such cases it is best to use distilled or deionized water in making up the reagent solution."

Ketostix

"Acetoacetic acid, if present in urine or serum, will form a colored complex with nitroprusside in the presence of aminoacetic acid. Sodium phosphate provides an optimum pH environment for this reaction.

"**Directions for detection of ketone bodies in urine:**

"1. Dip test end of strip in urine or briefly pass through urine stream.

"2. At 1 min. compare color of dipped end with color chart. (Color chart on bottle label.)

"**Interpretation:**

"*Negative*—Color of test strip remains unchanged or appears cream colored from wetting.

"*Positive*—At 1 min. test end of strip develops color varying from pink to purple, depending upon amount of ketone bodies present.

"**Directions for detection of ketone bodies in serum:**

"1. Dip test end of strip in plasma or serum.

"2. At 1 min. compare the color of dipped end with color chart. This procedure is adequate to detect most ketonemias and will reliably demonstrate 25 mg. acetoacetic acid/100 ml. serum or plasma. If a more sensitive test is desired, a 3 min. reading of Ketostix can be made, in which case amounts of acetoacetic acid as small as 10 mg./100 ml. can easily be detected.

"**Interpretation:**

"*Negative*—Color of test strip remains unchanged or appears cream colored from wetting.

"*Positive*—At 1-3 min. test end of strip develops color varying from pink to purple, depending upon amount of ketone bodies present."

Acetest

"Acetoacetic acid or acetone, if present in urine or blood, will form a colored complex with nitroprusside in the presence of aminoacetic acid. Sodium phosphate provides an optimum pH environment for this reaction and lactose enhances the color quality.

"**Directions for detection of ketone bodies in urine:**

"1. Place tablet on a clean surface, preferably a a piece of white paper.
"2. Put **1** drop urine on tablet.
"3. Take reading at **30 sec.** Compare color of tablet to color chart.

"**Interpretation:**

"*Negative*—Tablet color is unchanged or turns cream color.

"*Positive*—At 30 sec. color of tablet varies from lavender to deep purple. Results may be recorded as trace, moderate, or strongly positive.

"**Directions for detection of ketone bodies in whole blood:**

"1. Place **1** drop blood on Acetest tablet. (One of the great advantages of this test is that a drop of whole blood from a fingertip puncture may be used.)
"2. Allow the blood to clot on the tablet for approximately **10 min.**
"3. Lift off clot or wash off with water. Compare color of tablet to color chart.

"**Interpretation:**

"*Negative*—Tablet color is unchanged or turns cream color.

"*Positive*—At 10 min. color of tablet varies from lavender to deep purple. Results may be recorded as trace, moderate, or strongly positive.

"**Directions for detection of ketone bodies in serum or plasma:**

"1. Place **1** drop of serum on Acetest tablet.
"2. Readings made at **2 min.** will detect as little as 10 mg./100 ml. serum, and quantities greater than this will give a color within a shorter interval of time.

"**Interpretation:**

"*Negative*—Tablet color is unchanged or turns cream color.

"*Positive*—At 2 min. color of tablet varies from lavender to deep purple. Results may be recorded as trace, moderate, or strongly positive."

Hematest

"A positive reaction with Hematest is based upon the catalytic properties of hemoglobin. When the Hematest tablet is moistened with water, the reagents are washed down onto the filter paper. Tartaric acid and calcium acetate react with strontium peroxide to form hydrogen peroxide. Hemoglobin of the blood catalytically decomposes hydrogen peroxide with the liberation of oxygen, which oxidizes orthotolidine to a blue-colored derivative.

"**Directions for detection of occult blood in feces:** Hands, glass dropper, and working area must be clean and free of traces of blood.

"1. Smear a solid specimen of feces on filter paper. Do not use an emulsion. (See color photographs for example [included in package].)
"2. Place Hematest tablet in center of specimen.
"3. Place 1 drop water on tablet, wait 5-10 sec., and flow a second drop on tablet so that it runs down the sides onto the filter paper.

"**Interpretation:**

"*Negative*—If within 2 min. color does not appear on the filter paper around the tablet, test is considered negative.

"*Positive*—Diffuse blue color appears on the filter paper around the tablet within 2 min. The amount of blood is proportional to the time of appearance and to intensity of color. (Color on the tablet is of no significance.)

"**Directions for detection of occult blood in body fluids (urine, sputum, mammary secretions, and vaginal and uterine secretions):**

"1. For fluid specimens place 1 drop liquid on the filter paper. Place tablet in the center of moistened portion of the filter paper and proceed with directions and interpretations described above.

"**Positive reactions:** A positive reaction with Hematest is based on the catalytic properties of hemoglobin. Although no drug, including iron and bismuth, has been found to react with Hematest, certain plant residues and bacteria in feces contain reactive substances which may be present in a stool specimen in sufficient concentration to produce a color reaction with Hematest that is faint and slow in appearing. If a false positive reaction is [suspected], confirmation by examination of a second stool specimen is desirable.

"**Negative reactions:** False negative reactions are prevented by fixed sensitivity and stability of the Hematest reagent tablets. However, rarely a small amount of blood may be contained in only one portion of a stool specimen. Testing of several smears from a stool specimen may result in the detection of occult blood which might be undetected by a single test."

Occultest

"A positive reaction with Occultest is based upon the peroxidase properties of hemoglobin. When the Occultest tablet is moistened with water, the reagents are washed down onto the filter paper. Tartaric acid and calcium acetate react with strontium peroxide to form hydrogen peroxide. Hemoglobin of the blood catalytically decomposes hydrogen peroxide with the liberation of oxygen which oxidizes orthotolidine to a blue-colored derivative. The sodium bicarbonate and a small amount of the tartaric acid serve as an effervescent couple to facilitate solution of the reactive ingredients and thus increase the sensitivity of the test. A red dye is added to mask discoloration of the tablet.

"**Directions:** Hands, glass dropper, and working area must be clean and free of traces of blood.

"1. Place 1 drop well-mixed uncentrifuged urine in center of filter paper square.
"2. Put Occultest tablet in center of moist area.
"3. Place 2 drops water on tablet.
"4. A positive reaction is read on filter paper within 2 min.

"**Interpretation:**

"*Negative*—If within 2 min. color does not appear on the filter paper around the tablet, test is considered negative.

"*Positive*—Diffuse blue color appears on the filter paper around the tablet within 2 min. Amount of blood present is proportionate to intensity of blue color, its area on the filter paper, and rapidity of color development.

"**Positive reactions:** A positive reaction with Occultest is based upon the peroxidase properties of hemoglobin. Although no drug, including iron and bismuth, has been found to react with Occultest, certain bacteria contain reactive substances and may be present in an old urine specimen in sufficient concentration to produce a color reaction with Occultest that is faint and slow in appearing. If a false positive reaction is [suspected] confirmation by examination of a fresh urine specimen is desirable.

"*Note:* Occultest is designed to detect occult red blood cells or free hemoglobin in urine. Another Ames product, Hematest reagent tablets, which is somewhat less sensitive than Occultest, has been specifically designed to detect occult blood in feces."

Hemastix

"Hemoglobin of blood (or myoglobin) catalyzes the interaction of orthotolidine and peroxide, oxidizing orthotolidine to a bright blue color. In the absence of hemoglobin or myoglobin no color develops.

"**Directions:**

"1. Dip test end of strip into well-mixed urine or briefly pass through urine stream.
"2. At 1 min. compare test end of strip with color chart on the bottle label.

"**Interpretation:**

"*Positive*—Test end of strip turns blue and may be read as small, moderate, or large, depending upon the intensity of the color produced.

"*Negative*—No blue color is present at the end of 1 min."

Ictotest

"When 5 drops urine sample containing bilirubin are placed on the asbestos-cellulose mat, the absorbent qualities of the mat cause the bilirubin to remain on the outer surface of the mat. The tablet containing the reactive ingredients is then placed on the mat and 2 drops water are allowed to flow over the surface of the tablet. This allows a certain amount of the reactive ingredients of the slightly effervescent tablet to dissolve and to wash onto the surface of the mat. A chemical coupling of bilirubin with *p*-nitrobenzene diazonium *p*-toluene sulfonate occurs with the formation of a blue or purple color. The sulfosalicylic acid provides a suitably acid environment for this reaction to occur. The small amount of sodium bicarbonate along with a small portion of the sulfosalicylic acid provides an effervescent mixture which ensures solution of some of the tablet ingredients in the 2 drops water.

"**Directions:**

"1. Place 5 drops urine on one square of special test mat (supplied).
"2. Place Ictotest reagent tablet in center of moistened area.
"3. Flow 2 drops water on tablet.

"**Interpretation:**

"*Negative*—Mat shows no blue or purple color within 30 sec. (A slight pink or red color that may sometimes appear should be interpreted as negative.)

"*Positive*—Mat (around tablet) turns blue or purple. Amount of bilirubin present is proportionate to speed and intensity of color reaction."

Note: In a report by Ivy and Hurley,[29] this test was performed routinely on 25,000 patients over a period of 6 yr. Clinical icterus was observed in only 20% of the examinations which were positive by Ictotest.

Phenistix

"Ferric ions will react with phenylpyruvic acid in a properly acidified medium to form a characteristic green or gray to blue color. With

Phenistix, the necessary ingredients are impregnated into a stiff cellulose strip which is merely dipped into a test urine or pressed against a wet diaper to obtain a reaction. Ferric ions are supplied as ferric ammonium sulfate. Optimum acidity for the reaction is provided in the form of cyclohexylsulfamic acid. Magnesium ions in the form of magnesium sulfate are incorporated into the formulation to prevent the interference of urine phosphates in the color reaction. The composition is such that a minimal interference is obtained from other possible urine constituents which give colored reactions with ferric ions.

"**Directions:**

"1. Dip test end of strip in urine and remove immediately or moisten by pressing against wet diaper.

"2. Compare color of dipped end with color chart at ½ min. (Color chart on bottle label.)

"**Interpretation:**

"Negative—If within ½ min. color does not appear on the test end of the strip, test is negative.

"Positive—A color change ranging from gray to blue-gray appears on the test end of the strip within ½ min. If the color of the test strip closely matches a color on the color scale, the concentration of phenylketonuria is so recorded. If the color of the test strip lies between two colors of the color scale, the concentration of phenylketonuria may be indicated accordingly (e.g., between 40 and 100 mg./100 ml.) or interpolated (e.g., approximately 60 mg./100 ml.).

"**Positive reactions:** Theoretically large amounts of ketone bodies present in the urine, as in very severe ketosis, might produce an atypical color reaction with Phenistix. However, as the color is atypical, it is unlikely that it will cause confusion. Furthermore, this atypical color reaction has been observed only under laboratory conditions (ketone bodies added to urine), and it is unlikely that such high concentrations of ketone bodies would occur under conditions of clinical practice.

"Salicylates and metabolites of phenothiazine derivatives impart a pink color to Phenistix which is differentiated readily from the gray to blue-gray colors produced by phenylpyruvic acid. (With ferric chloride solutions these differentiations may be difficult. Furthermore, ferric chloride is much more sensitive to phenothiazine metabolites than is Phenistix.)

"Phenistix will react to *p*-hydroxyphenylpyruvate, but the color is fleeting and should not be a problem if the test is read at ½ min. as recommended.

"High concentrations of bilirubin in the urine may alter the normal Phenistix color reaction.

"**Negative reactions:** The normal range of the pH of urine does not significantly affect Phenistix. However, with old decomposed ammoniacal urine specimens interference with the color reaction may occur. Therefore, old urine specimens should not be used with Phenistix.

"It must be kept in mind that with all test procedures, including highly sensitive tests such as Phenistix, factors such as dietary intake, severity of the existing condition, and dilution of the urine may result in undetectable concentrations of the substance tested. For these reasons, in a case of a negative Phenistix reaction, it may be desirable to test several specimens at the time testing is scheduled."

Dextrotest

"**P tablets:** The sodium bicarbonate and a small amount of sulfosalicylic acid act as an effervescent couple to effect rapid solution of the tablet in water. One P tablet dissolved in 2 ml. water provides a strong sulfosalicylic acid solution which will completely precipitate the protein present in 1 ml. blood. Filtration of the sulfosalicylic acid-blood mixture yields a protein-free filtrate necessary for estimation of the reducing sugars.

"**S tablets:** One S tablet added to 1 ml. protein-free filtrate obtained with P tablet as just described provides a means of estimation of the amount of reducing sugars present. The sodium carbonate and a small amount of citric acid act as an effervescent couple to ensure rapid solution of the tablet. Reducing substances present in the filtrate react with the copper sulfate to reduce the cupric ions to cuprous oxide. The solution turns from blue through green to reddish brown, depending upon the amount of reducing sugars present. Sodium hydroxide provides the alkaline medium for this reaction, and the heat required is provided both by the solution of the sodium hydroxide and by the reaction between sodium hydroxide and citric acid.

$$2\ \mathrm{Cu}^{++} + \underset{\text{sugar}}{\text{Reducing}} \xrightarrow[\text{alkali}]{\text{Heat and}} \mathrm{Cu_2O} + \underset{\text{sugar}}{\text{Oxidized}}$$

"**Directions:**

"1. Place water in narrow graduated test tube P to mark 1 (2 ml.) and add one reagent tablet P. While tablet P is dissolving, fold filter paper and place dry on test tube S.

"2. After reagent tablet P dissolves, add blood (1 ml.) to mark 2 on test tube. Invert and shake to mix contents thoroughly.

"3. Immediately pour brown mixture from tube P through dry filter into tube S. (Filter cone will hold entire contents of tube P.) Collect filtrate to mark.

"4. Add 1 reagent tablet S to filtered solution. Do not shake while boiling. After boiling ceases, shake test tube briskly 3 or 4 times, making certain that the foam is carried down into the solution. Wait 30 sec. and compare [with] color chart.

"**Interpretation:** The color scale provided with the Dextrotest kit has 3 color blocks varying from blue-green to green to orange-brown, representing, respectively, blood sugar levels of 100 mg./100 ml., 150 mg./100 ml., and 200 mg./100 ml.

"*Note:* If test indicates blood sugar levels above 200 mg./100 ml., the test may be repeated using 2.5 ml. water, 1 tablet P and 0.5 ml. blood. The same amount of filtrate is collected, and 1 tablet S is used, as described. Blood sugar concentrations read from the chart are multiplied by 2.

"**Precautions:** Dextrotest tablets (P and S) are hygroscopic but are stable indefinitely provided the foil packaging is not punctured or torn.

"*P tablets:* These effervescent tablets are somewhat hygroscopic. As long as the tablets dissolve to give a clear solution, they are satisfactory.

"*S tablets:* These tablets are very hygroscopic and should be protected from exposure to moisture, even the humidity of the air. There is one special precaution that should be observed in handling. Tablet S contains sodium hydroxide and therefore should not be handled by anyone having wet or damp hands. The tablets are also sensitive to heat. At 120° F. or above the tablets will deteriorate in a short time. The appearance of the tablets reflects their condition. Tablets which are bluish white and slightly spotted will still react satisfactorily. However, if the tablets show blue or black discoloration in spots or in overall appearance, they will not react satisfactorily."

Chapter 101

Microscopic examination

SAM FRANKEL

URINARY SEDIMENTS

There are 2 general types of urinary sediments: organized and unorganized.

Organized sediments include (1) casts—(hyaline, epithelial, granular, blood, waxy, mixed, cylindroids, and false or pseudocasts.) (2) epithelial cells, (3) leukocytes or pus cells, (4) erythrocytes, (5) spermatozoa, (6) urethral filaments, (7) tissue debris, (8) animal parasites, (9) fibrin, (10) microorganisms, and (11) foreign substances in urine.

Unorganized sediments include (1) crystals (ammonium magnesium phosphate [triple phosphate], calcium oxalate, calcium phosphate, calcium sulfate, calcium carbonate, uric acid, cystine, cholesterol, hippuric acid, leucine, tyrosine, and sulfonamides) and (2) urates.

Preparation of sediment

1. Pour the urine to be examined into a 15 ml. conical centrifuge tube. Centrifuge at moderate speed for 3-5 min.
2. Pour off the supernatant fluid. The sediment remains in the tube. Shake the tube to mix the sediment. Use a dropper with rubber bulb. Draw up a drop of sediment in the dropper and place in the center of a glass slide. Place a clean cover glass over the drop. Take care to eliminate bubble formation.
3. Make a survey examination under low magnification to detect the presence of casts, and to determine quantities of cells and crystals present. Then examine under high dry magnification to make a close study of the contents of the specimen.

If a bacteriologic examination is ordered, the procedure is different (see Chapter 31).

Organized sediments

Casts

Tubular casts were originally found in the urine by Henle in 1842. In 1867 Rovida gave a thorough account of their nature and formation. Casts are probably the products of an albuminous exudate from the blood vessels with addition of swollen and destroyed epithelia. In almost all cases in which casts are present albumin is found in moderate or large amount, but there are undoubtedly cases in which the amount of albumin is small or may be entirely absent.

The appearance of casts in the urine is always of the highest diagnostic importance. Hyperemia of the kidneys may suffice to produce casts in the urine, and they can at times be found in small numbers when the kidneys are perfectly normal. They have been described in cases of gastrointestinal inflammation, in jaundice, and in acute and chronic anemia, as well as in nervous affections of different kinds, without any inflammation of the kidneys. In general it may be said that in the majority of cases the presence of tube casts

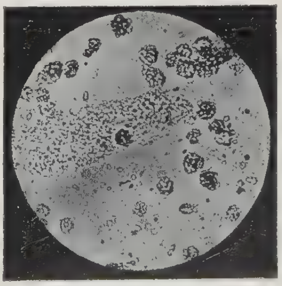

Fig. 619. Pus cells and finely granular cast. (×400.)

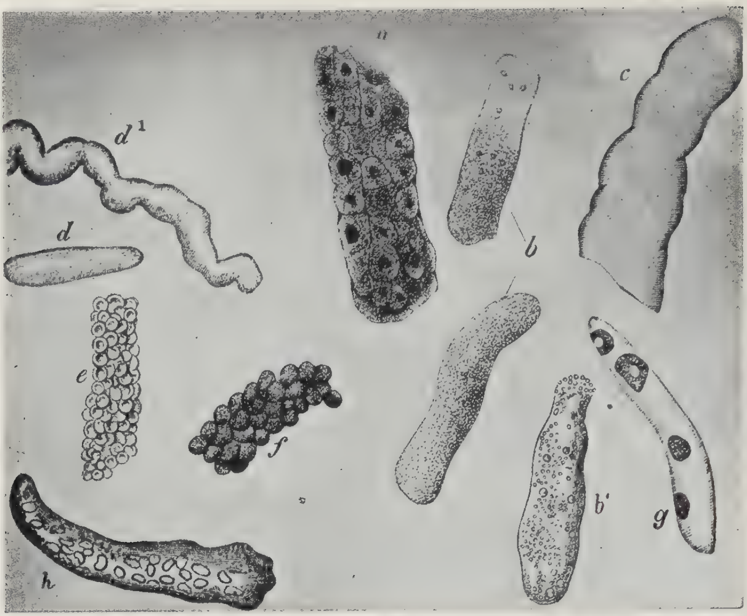

Fig. 620. Urinary casts (Bizzozero). **a,** Epithelial cast; **b** and **b**¹, nuclei; **c,** waxy cast; **d,** hyaline cast; **d**¹, convoluted hyaline cast; **e,** blood cast; **f,** pus cast; **g,** hyaline casts with epithelial and white cells; **h,** waxy cast with cells. (From Sahli, H.: Lehrbuch der klinischen Untersuchungsmethoden für Studierende und praktische Aerzte, Leipzig, 1931, Franz Deuticke.)

signifies the presence of nephritis, but small numbers of hyaline casts may be found in renal congestion unaccompanied by an inflammation.

Casts have been divided in many different ways, but perhaps the simplest is to divide them into **true** casts and **false** casts. The former denote the presence of nephritis, but the latter are accidental formations.

Generally speaking, hyaline, epithelial, and blood casts are found in acute nephritis, whereas granular, fatty, and waxy casts are found in the chronic conditions.

All true casts may appear in 3 distinct sizes, according to the portion of the uriniferous tubules from which they originate. The narrowest casts are those formed in the narrow tubules and the next in size are from the distal convoluted tubules, whereas the largest are always formed in the straight collecting tubules. Casts from the proximal convoluted tubules, those directly arising from the capsule of the tuft, never appear in the urine since they cannot pass the narrow tubules. A certain prognostic value is attached to the size of the casts. The mildest degrees of the disease are indicated by casts from the narrow tubules and a small number of casts from the convoluted tubules. Occasionally, pedunculated casts are found; that is, formations from the place of transition of the narrow tubules into the distal convoluted tubules. Casts of all 3 sizes, the largest arising from the straight collecting tubules, permit a conclusion of chronic nephritis in the whole organ, and upon this condition a very unfavorable prognosis can be established.

Repeated examinations of urine, especially from a mixed 24 hr. voiding, should always be made before an opinion as to the prognosis of a case can be of any value. All the features, chemical, microscopic, and physical findings, must be taken into consideration.

Hyaline casts: Hyaline casts are pale transparent formations of variable length and sometimes of considerable size and are the most difficult forms of renal casts to detect under the microscope. Those from the convoluted and straight collecting tubules are usually more or less regular though the latter may be very broad; those from the narrow tubules are occasionally tortuous or spiral and at times exceedingly narrow and delicate. Different formations, such as pus cells and fat globules, may be seen upon them in small numbers but are accidental and do not change the diagnosis. When casts are very delicate and pale, it is advisable to color the casts by the addition of a drop of iodine-potassium iodide solution (iodine, 1 part; potassium iodide, 2 parts; water, 300 parts) upon the slide, which will stain them yellow and render them more distinct. (See p. 1848 for a stain for urinary casts.)

Epithelial casts: Epithelial casts, when present, always denote an acute process; the more pronounced it is, the larger is the number of these casts. They vary in size according to their origin but are never as long as some hyaline casts and are usually quite regular. The number of epithelia seen upon these casts varies considerably. Sometimes no more than 2, 3, or 4 will be found upon a cast, whereas at other times the cast is completely filled with them, though still showing its structure plainly. Those from the convoluted and narrow tubules contain spherical or slightly irregular epithelia, whereas those from the straight collecting tubules usually contain a number of columnar epithelia. Occasionally these casts are of a yellowish color with a slightly increased refracting power because of their imbibition of the coloring matter of the blood. As long as the nephritis is acute, the epithelial casts will have this appearance. As soon as the inflammation enters the subacute or chronic stage, their character changes, and fat globules appear. We can then no longer consider them pure epithelial casts.

Granular casts: Granular casts rarely appear in strictly acute inflammations. They are perfectly regular and have sharply defined contours, or they are more or less curved or appear curved at one side while they are straight at the other. Their ends are either rounded or partly broken, and they may be broader at one place and narrower in another —a peculiarity especially pronounced in those from the narrow tubules. The granular material of which they are composed consists of albumin, epithelial cells, fat, or disintegrated leukocytes or erythrocytes. Their granulations vary from fine to coarse. They may be coarsely granular at both ends and finely granular in the center or finely granular above and below and coarsely granular in the center. They are due to a disintegration of the kidney epithelia. In cases of long duration the granules are changed into glistening fat granules and globules.

Blood casts: The appearance of blood casts in the urine reveals acute diffuse nephritis, acute congestion of the kidney, or renal hemorrhage. Their appearance varies greatly —they are usually more irregular than the epithelial casts, and their ends are more or less rounded. They may be studded with a varying number of red blood cells without change in color. If they are retained in the tubules for some time, the blood cells lose their shape, and the casts take on the appearance of dark, rust-brown granular clusters. Besides these, conglomerations of fibrin, the so-called **fibrin casts,** are occasionally found, but properly speaking they are not true casts. In the rare cases of hemoglobinuria, irregular dark casts, which appear granular and are composed of disintegrated blood corpuscles—the so-called **hemoglobin casts**—may be quite abundant.

Waxy casts: The chemical nature of waxy casts is different from that of hyaline casts. Waxy casts are characterized by wavy, fluted contours, a high refracting power, a more or less yellowish color, and a high degree of brittleness. They vary in size and are always more or less irregular, sometimes resembling regular corkscrew windings. These casts appear only in chronic processes and never in acute inflammations. Pure waxy casts may be found at times studded with different formations, which, of course, do not change the character of the cast. At times they are extremely large and may then be almost entirely broken in different portions.

Mixed casts: Casts in some cases do not appear in their true form but may be more or less mixed. Any 2, 3, or 4 varieties may be so intermingled as to be difficult of differentiation. There may be an epithelial-granular fatty variety, with the epithelia perfectly intact; combinations of waxy casts, fatty and waxy, granular-fatty and waxy, or blood and waxy casts.

Other casts (cylindroids): Besides these varieties, mucus casts or cylindroids are occasionally found among the true casts. They may contain a varying number of fat globules, but their striated, irregular appearance is sufficient to clarify the diagnosis.

False casts or pseudocasts: These casts have no connection whatever with diseases of the kidney. They are conglomerations of different substances upon mucus threads or casts or accidental formations in the shape of casts. Among these we designate the urate casts which are conglomerations of urates. Ammonium urate casts are described as occurring in infants and forming small, reddish brown masses; they may also be seen in adults, although very rarely. Sodium urate masses, resembling casts, may be mistaken for granular casts, but they have the characteristic yellowish brown color of sodium urate and show no outlines. We sometimes see a formation composed of granules of sodium urate changing to globules and dumbbells.

Among the other **pseudocasts** the more common are bacterial, pus, fat, and fibrin casts.

Bacterial casts: These casts occur frequently, especially when the urine has been allowed to stand in a warm room for 12 hr. or more so that a large number of bacteria have developed. They vary considerably in size, but their outlines are pale and more or less irregular. They are composed of masses of micrococci, not of granules. In order to clarify their diagnosis, it may in rare cases be necessary to add 1 or 2 drops of some strong mineral acid or alkali, to which they will be seen to have a great resistance.

Pus casts: Pus casts are sometimes found. The pus cells may be massed together, with no outlines visible, or they may be more loosely arranged and may contain a number of small fat globules. They indicate renal suppuration.

Fat casts: Fat pseudocasts are rare but they have been found in a few cases of so-called lipuria. They consist of large fat globules, of a very high refraction, and occasionally contain margaric acid needles.

Fibrin casts: These casts are found in cases of hemorrhage. They are large, have irregular, more or less sharply defined contours, and are of a yellowish or yellowish brown color.

Stain for urinary casts

The technic of Behre and Muhlberg[30] is very useful in the study of the structure and composition of urinary casts.

Reagents:

1. Aqueous solution eosin Y, 0.5%.
    (a) Dissolve 500 mg. eosin Y in 100 ml. distilled water.
2. Methyl blue-picric acid solution.
    (a) Add 1 ml. 1% aqueous solution of methyl blue to 10 ml. saturated picric acid solution. Use Merck reagent picric acid.
    (b) Add 10 drops glycerol to each 10 ml.*

Procedure:

1. Centrifuge the urine and decant the supernatant liquid.
2. Add to the sediment 1 drop eosin solution and mix by shaking from side to side.
3. After 1 or 2 min. add 2 drops methyl blue-picric acid solution and mix. The color of the sediment should be distinctly blue-green. If it is reddish brown, add more of the blue solution until the blue-green color is obtained but do not add too much.
4. Transfer some of the stained sediment to a glass slide, cover with a cover glass, and examine under the microscope.

The quantities of the stains added to the urine may be varied according to the amount of sediment. If the cells are not red enough, add more eosin to the sediment. Enough methyl blue should be in the stain to impart a distinct blue color to the casts, but too much stain will make them too dark.

To make permanent mounts, add more glycerol to the sediment and seal the edges of the cover glass with balsam.

By the use of this staining method, hyaline casts are stained a clear blue of varying intensity. The more irregularly shaped bodies, sometimes classed as cylindroids, are similarly stained. An irregular distribution of material, a mealy structure, or a striated appearance sometimes appears in bodies which seemed perfectly homogeneous before staining. Mucus threads and amorphous mucous material are also stained blue, making the differentiation from castlike bodies very evident. Mixed, finely granular casts present a striking picture of fine, dark granules powdered over the light blue hyaline body. Some coarsely granular casts are stained deep blue, the granules of others are yellow, orange, or dark reddish brown. Renal epithelial cells are usually red, sometimes orange or yellowish. Red blood cells are stained a brighter red. Pus cells are usually red, occasionally blue; epithelial cells from the urinary passages are either red or blue. Fat globules are unstained. In cells undergoing fatty degeneration, the fat globules are seen strikingly against reddish cellular material.

By the use of this stain, possible differences in structures otherwise undifferentiated may be made out. In general, the method deserves adoption and further investigation.

The preparation of permanent slides of urinary sediment has been described by Dixon and Holmes.[30a] Using a preserving medium of gelatin, glycerin, phenol, and water they have been able to retain slides in good condition for several years.

Interpretation of casts in urinary sediment: It is known from life insurance statistics that casts, either hyaline or granular and either intermittent or constant, may herald the onset of cardiovascular renal disease about 10 yr. before the cardiovascular renal apparatus is seriously affected. Too much attention, therefore, cannot be paid to the microscopic examination of urinary sediment.

The finding of casts of various types in urinary sediment provokes the question of just what significance should be attached to this finding. First, in order to answer the question the types of casts must be discussed separately. Hyaline casts, for instance, are often found in urine of healthy people after exercise. Their presence does not always parallel the quantity of albumin in cases of renal albuminuria. During recovery from acute nephritis, they may be present for some time after albuminuria has disappeared. Hyaline casts are frequently found in jaundice without any accompanying albuminuria.

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*Instead of methyl blue solution, 1.5 ml. of blue-black fountain pen ink added to 10 ml. saturated picric acid may be used. The dye used in these inks is evidently similar to methyl blue. The results with ink solutions are almost as good as those with methyl blue.

The presence of granular casts is always indicative of a severe degenerative process in the tubular epithelium. One may expect, however, to find isolated granular casts without severe renal disease as in chronic passive congestion or febrile albuminuria.

Epithelial casts have the same significance as granular casts; namely, isolated single casts are of little significance, but a large number indicates renal degeneration.

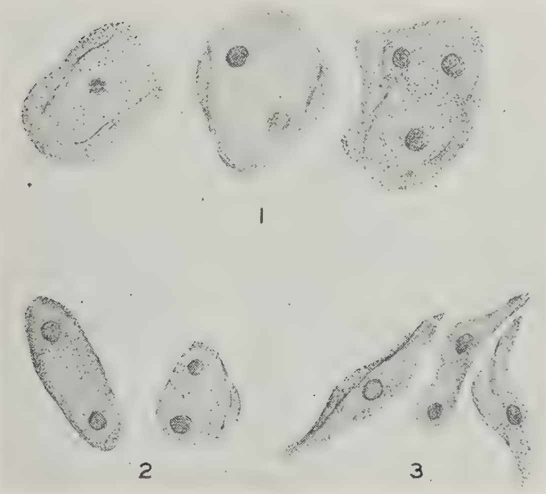

Fig. 621. Epithelia from vagina. **1**, Upper layer; **2**, middle layer; **3**, deepest layer.

Fatty casts are found in the later stages of severe acute nephritis and the subacute and chronic stages of the disease, as well as in the nephroses.

Waxy casts are found whenever there are severe degenerative changes in the kidney existing over a long period of time. At one time they were believed characteristic of the amyloid kidney, but this has been disproved.

Hemoglobin casts have been found in the presence of hemoglobinuria.

Cylindroids are found in inflammations of the urinary passages, especially in acute disturbances of the renal circulation, such as occur from embolus formation in the renal artery.

In summary, granular, epithelial, fatty, and lipoid casts indicate degenerative changes in the tubular epithelium.

Epithelial cells

There are 3 kinds of epithelia: (1) flat or squamous, (2) cuboidal, and (3) columnar or cylindrical.

Squamous epithelial cells are large, are more or less irregular in outline, and exhibit a broad front surface, whereas in edge view they are narrower and somewhat spindle-shaped. They are granular and possess one or more nuclei. Occasionally a nucleus drops out, leaving a vacuole.

Cuboidal epithelia have about the same diameter in all directions, whereas **columnar epithelia** are elongated in one direction. Columnar epithelia may be ciliated, having one or more delicate hairlike prolongations on the outer surface. All epithelia are granular and possess one or more nuclei.

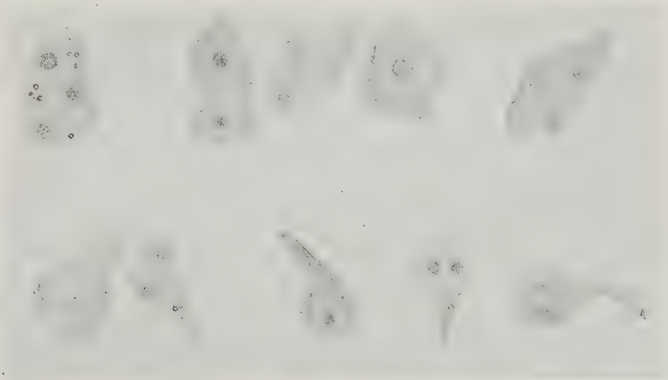

Fig. 622. Epithelia from urethra.

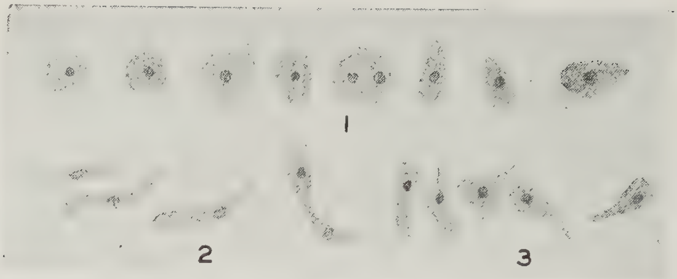

Fig. 623. **1,** Epithelia from prostate gland; **2,** epithelia from ejaculatory ducts; **3,** epithelia from seminal vesicles.

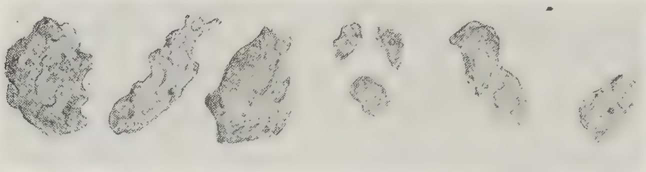

Fig. 624. Epithelial scales.

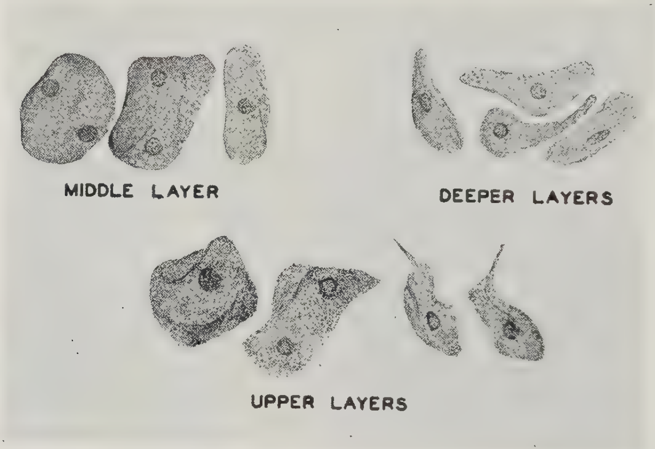
MIDDLE LAYER
DEEPER LAYERS
UPPER LAYERS

Fig. 625. Epithelia from bladder.

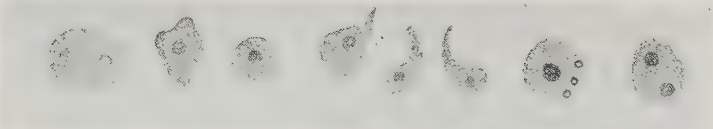

Fig. 626. Epithelia from ureters.

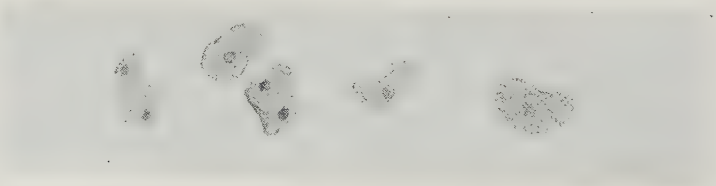

Fig. 627. Epithelia from pelvis of kidney.

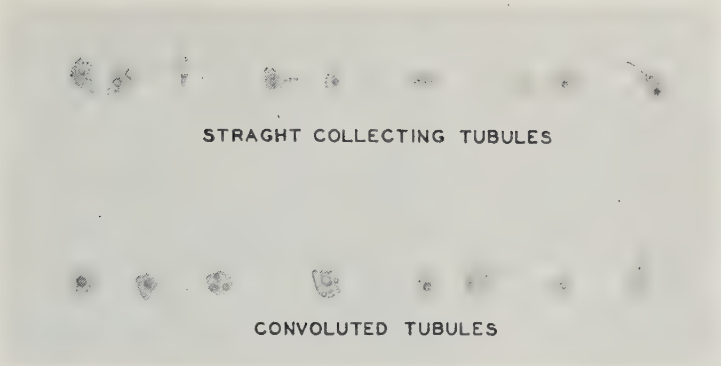

Fig. 628. Epithelia from uriniferous tubules.

Epithelia may occur in a single layer or may be stratified. They change in appearance to a certain extent in urine.

Epithelia from the **bladder** are of 3 distinct types; those from the upper layers are flat or squamous; those from the middle layers are cuboidal; those from the deepest layer are columnar. Squamous epithelia are insignificant in the urine except when they appear along with pus cells.

Epithelia from the **pelvis of the kidney** are smaller than those from the bladder but larger than those from the ureters. The majority of the pelvic epithelia are caudate, pear-shaped, or lenticular, although they are sometimes quite irregular. The regular cuboidal shapes, smaller than those from the bladder, are less numerous.

Epithelia from the **ureters** are rarely found alone but usually with those from the pelvis of the kidney. They are round, globular, or slightly irregular when seen in urine and are distinctly smaller than those from the pelvis. They closely resemble the epithelia from the prostate gland. Occasionally small columnar epithelia from the deeper layers are found.

Epithelia from the **uriniferous tubules** of the kidneys are very important. They are of 2 kinds: the cuboidal from the convoluted tubules and the columnar from the straight collecting tubules. They are distinctly smaller than either those from the pelvis of the kidney or from the ureter in the same case, though their sizes vary to a certain degree in different cases. They are round, globular, or slightly irregular. They are about ⅓ larger than pus cells.

Leukocytes or pus cells

Leukocytes or pus cells are larger than erythrocytes and smaller than renal cells. They are about the same size as or slightly larger than the nuclei of the large squamous epithelial cells. They contain one or more nuclei which are rendered distinct by the addition of acetic acid. They are dull grayish, and many are granular.

Erythrocytes

Erythrocytes appear in the urine as normal biconcave or crenated disks, usually shiny and light-refractive. They are smaller than pus cells, have no nuclei, and dissolve in acetic acid. They must not be confused with yeast cells.

The pathologic conditions in which erythrocytes are found in the urinary sediment are as follows: hemorrhage of the kidney, hemorrhage of the urinary tract, hemorrhage from congestion, traumatic hemorrhage, and hemorrhagic diathesis.

Spermatozoa

Spermatozoa appear after coitus or in the following conditions: diseases of the genital organs, nocturnal emissions, and epileptic and other convulsive attacks. They may or may not be motile. They have an oval body and a long delicate tail.

Urethral filaments

These peculiar threadlike bodies may be found in normal urines and also in the following pathologic conditions: acute gonorrhea, chronic gonorrhea, and urethrorrhea. These filaments are generally macroscopic. The first morning urine is the best specimen for filaments.

Tissue debris

The finding of fragments of tissue may sometimes be of assistance in defining a pathologic condition. These tissues may be found in the following pathologic conditions: tubercular

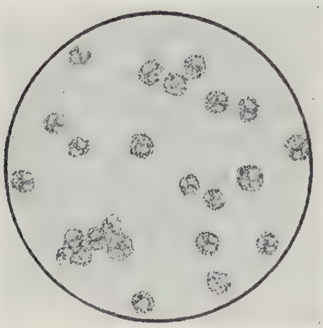

Fig. 629

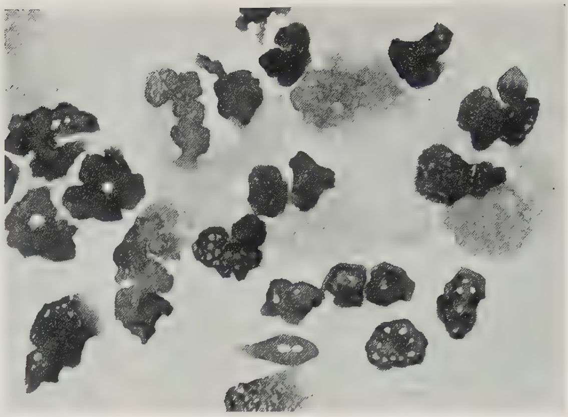

Fig. 630

Fig. 629. Pus cells in urine (unstained). (×400.)

Fig. 630. Pus cells in urine (stained). (×950.)

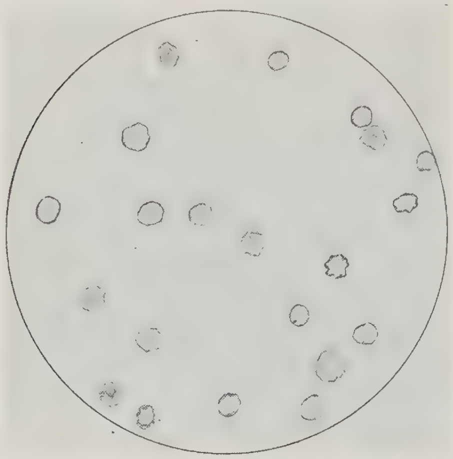

Fig. 631

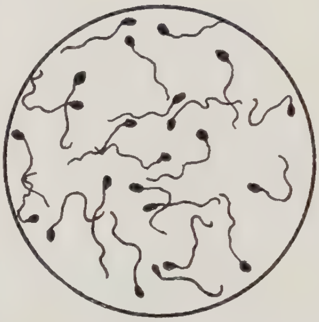

Fig. 632

Fig. 631. Erythrocytes in urine sediment (unstained). (×400.)

Fig. 632. Schematic drawing of human spermatozoa (unstained). (×400.)

affections of the kidney, tubercular affections of the urinary tract, tumor of the kidney, and tumor of the urinary tract. It is necessary, however, to make a histologic examination of these tissue fragments before coming to a final conclusion as to their origin.

Fibrin

Fibrin clots are occasionally found in the sediment of urines following hematuria.

Microorganisms

Fresh urine does not contain any organisms and is usually considered sterile when obtained directly from the bladder, but it may be contaminated when voided by bacteria present in the urethra or vagina. Bacteria develop in a short time in urine when it is allowed to stand and are present in large numbers in pathologic conditions. Such urine is turbid, and the condition is called bacteriuria.

Microorganisms in urine may be nonpathogenic or pathogenic. The nonpathogenic microorganisms found in urine are the mold fungi, such as *Candida, Penicillium glaucum,* or one of the aspergilli.

Yeast fungi are found in acid urine, especially in urine containing sugar. They consist of variously sized globules or cells. In the larger globules the process of budding can be seen. The most common organism found in urine is the *Micrococcus ureae.* This coccus causes ammoniacal decomposition of urine, urea being transformed by it into ammonium carbonate.

See Chapter 31 for complete bacteriologic examination of urine.

Animal parasites

The most common animal parasite found in the urine is the *Trichomonas vaginalis.* It frequently causes symptoms (vaginitis, cystitis). It is an oval organism with a tail-like extremity, with rapid motility.

Echinococci are occasionally found in urine and may have developed directly in the urinary organs or may have ruptured into them from some neighboring organ. The characteristic parts of the echinococci found in the urine are the hooklets as well as portions of the membrane. Scolices may also be found. These scolices are small and round and are supplied with a wreath of hooklets. When echinococci are found in urine, there is also evidence of hemorrhage or ulceration or both. Red cells are numerous, together with epithelia and connective tissue shreds as well as pus cells. The eggs of the parasite *Schistosoma haematobium* are found in the urine but not the parasite itself. The eggs are oval or flasklike in shape and taper considerably at one extremity, the other being rounded.

Filaria appears in the urine at times, causing severe hematuria or chyluria. When a milky urine, indicating chyluria, is found, an examination for filaria must be made. In examining urine for filaria, it is advisable to take the first urine voided in the morning since it is a well-known fact that the parasite is active in the night *(Filaria nocturna* or *Wuchereria bancrofti)* or during the resting hours of the patient, whereas it is quiescent during the working hours and cannot be found.

Ascaris lumbricoides, a common worm in children, may be found in the urine, having passed into the bladder through the urethra. Other parasites which may possibly be found in the urine are *Strongylus gigas, Enterobius vermicularis,* and *Cercomonas urinarius.*

Refer to p. 1011 for a discussion of parasites.

Foreign substances in urine

Care must be taken not to overlook foreign substances in the urine which may be mistaken by beginners for pathologic changes in the body. These substances are starch granules, hair, feathers, muscle fibers, particles of fat, fibers of silk, cotton, wool, etc. The presence of these materials may be due to many causes, such as exposure to air, pouring the urine into bottles which are not perfectly clean, the use of salves or dusting powders for the genital organs, or admixtures of particles from the feces. Human hairs are not infrequently found in the urine and may be known by the flat epidermal scales, firmly attached to each other, which form the main mass of the hair and by the varying amount of pigment. Feathers may appear in branching

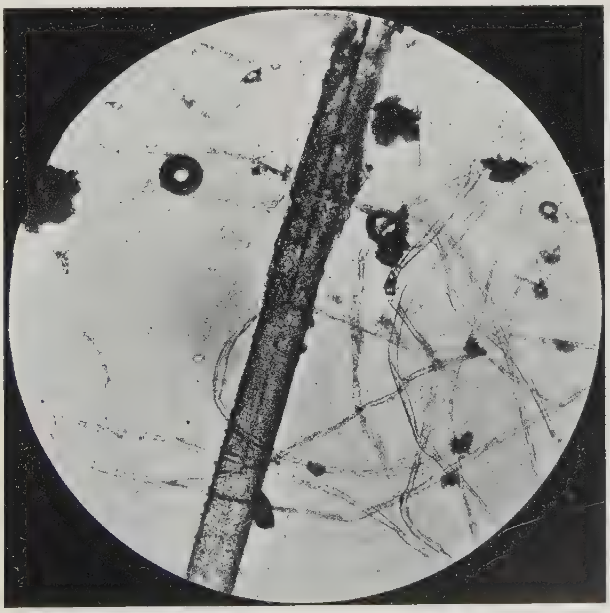

Fig. 633. Artifacts—hair, air bubble, cotton threads, soot. (×200.)

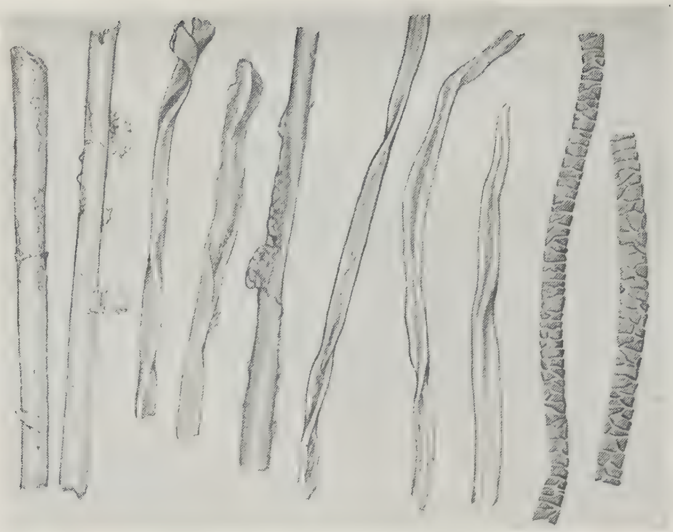

Fig. 634. Fibers—silk, linen, cotton, and wool.

formations. Scales from the wings of insects such as moths may also be found. They are more or less delicate serrated plates with a stemlike projection and vary considerably in length and breadth.

Starch globules are frequently seen in the urine. They are round or oval, are highly refractive, and vary greatly in size. The three most frequently found in the urine are rice starch, cornstarch, and wheat starch. Rice starch shows oblong even globules of medium size. Cornstarch is smaller, irregular, and at times almost hexagonal and contains an irregular hilum. Wheat starch consists of large globules as well as of small irregular formations in which the hilum may be entirely absent or is present only in the form of a dot.

Lycopodium, similar to starch and used as a dusting powder, consists of globular formations of different sizes, with a distinct shell; these globules are sometimes found in urine. Cellulose occurs in the urine in a variety of forms, sometimes in small and sometimes in large masses. A common variety of cellulose found in urine is cork occurring as single cells and having a yellowish brown color. Oil globules and air bubbles are commonly found in urine. They may be very large or extremely small, are either perfectly round or irregular, are of high refraction, and can frequently be differentiated by their yellowish color.

Flaws in glass as well as scratches in the cover glass may lead to mistakes in diagnosis. Flaws are irregular in size and shape and frequently resemble the wings of a butterfly. They show a faint blue refraction and are usually pale. Rust particles in both the cover glass and slides also occur and are larger or smaller dark or rust-brown irregular masses which must not be mistaken for coloring matter in the urine. Vegetable matter of different forms may be found in the urine as an admixture from the feces. Normal feces may occasionally be found mixed with urine, and their constituents must be known. If they are present and their accidental admixture can be excluded, the diagnosis of a fistula can be made. Among the particles seen from these sources are partly digested muscle fibers with many striations plainly visible, small numbers of connective tissue shreds from the meat diet, and mucus threads and mucus corpuscles.

Unorganized sediments

Crystals

Ammonium magnesium phosphate (triple phosphate) crystals: These crystals are found when the urine has undergone alkaline fermentation, either before or after being voided, and are crystallized in 2 forms: prisms and star-shaped, feathery crystals. These crystals may rarely appear in amphoteric or faintly acid urines, provided that the ammonium salts are present in large enough quantity. The pathologic conditions in which these crystals are frequently abundant are as follows: retention of urine in the bladder, paraplegia, chronic cystitis, enlarged prostate, and chronic pyelitis.

Calcium oxalate crystals: These crystals appear in the urinary sediment in at least 2 forms, i.e., octahedral and dumbbell. They

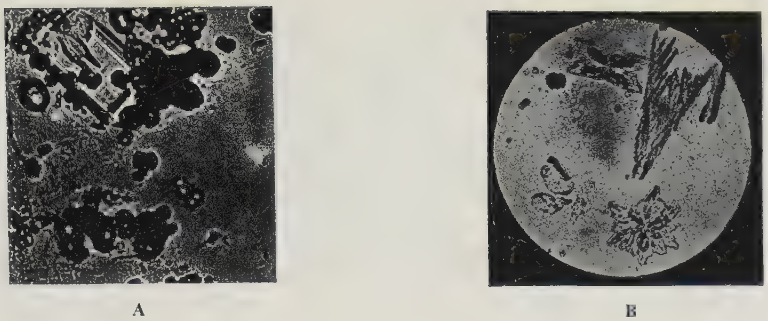

Fig. 635. **A,** Triple phosphate and ammonium urate crystals (×400). **B,** Incomplete triple phosphate (×400).

may be found in acid, neutral, or alkaline urines but are most frequently found in acid urines. Calcium oxalate crystals are found in normal urines but are increased in the following pathologic conditions: diabetes mellitus, organic diseases of the liver, diseases of the heart, and diseases of the lungs.

These crystals are found in the urine after ingestion of tomatoes, garlic, rhubarb, oranges, asparagus, etc.

Calcium phosphate (stellar phosphate) crystals: These crystals may occur in the urine in the amorphous, granular, or crystalline forms. They are wedge-shaped and often appear in rosette arrangements. They are sometimes mistaken for sodium urate but may be distinguished from the latter by adding acetic acid. Acetic acid will readily dissolve the phosphate, whereas the urate is much less soluble. The pathologic conditions in which calcium phosphate crystals are abundant are as follows: retention of urine in the bladder, paraplegia, chronic cystitis, enlarged prostate, and chronic pyelitis.

Calcium sulfate crystals: These crystals are very rarely seen and are found only in acid urines. They appear as long, thin, colorless needles or prisms and may be mistaken for calcium phosphate. They are readily distinguished, however, by the fact that calcium sulfate crystals are readily soluble in acetic acid. Calcium sulfate crystals are of practically no clinical importance.

Calcium carbonate crystals: These crystals are almost always in alkaline urine but may occur in amphoteric or faintly acid urine. They very frequently appear in the dumbbell shape and can be differentiated from calcium oxalate inasmuch as they dissolve in acetic acid with the evolution of carbon dioxide gas, whereas calcium oxalate remains unchanged in acetic acid.

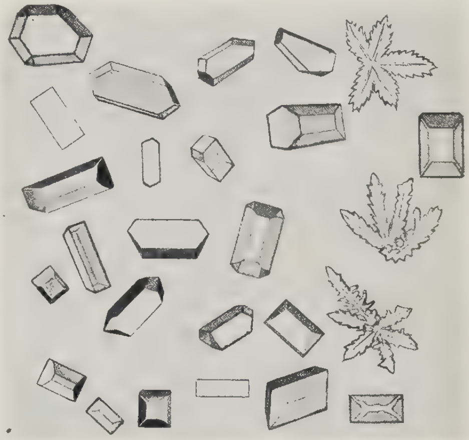

Fig. 636. Triple phosphate crystals (ammonium magnesium phosphate).

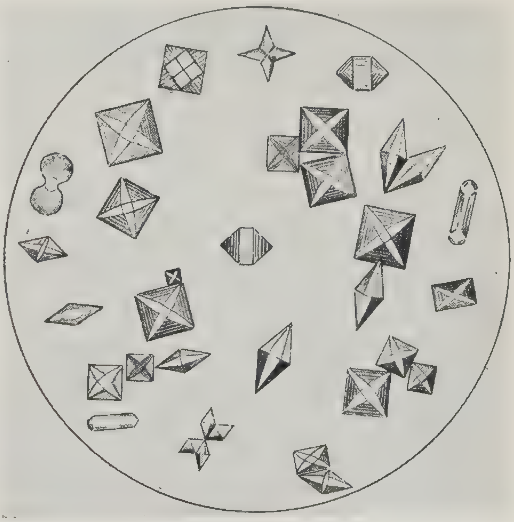

Fig. 637. Calcium oxalate crystals.

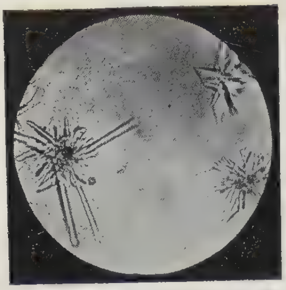

Fig. 638

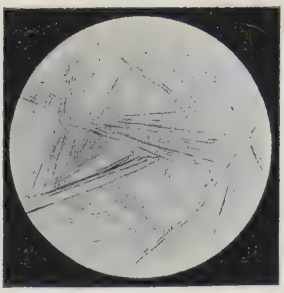

Fig. 639

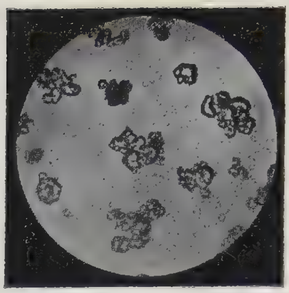

Fig. 640

Fig. 638. Calcium phosphate crystals. (×400.)

Fig. 639. Calcium sulfate crystals. (×400.)

Fig. 640. Calcium carbonate crystals. (×400.)

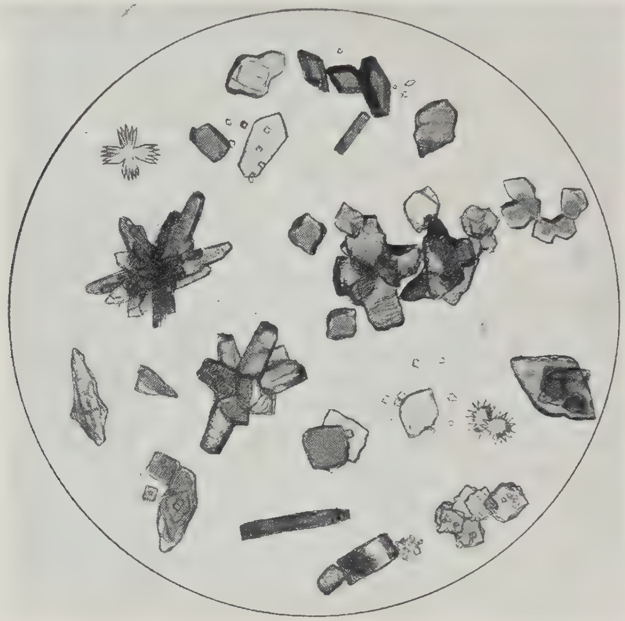

Fig. 641. Uric acid crystals.

Uric acid crystals: These crsytals appear in acid urines in the following forms: wedge-shaped, dumbbells, rhombic prisms, whetstones, prismatic rosettes, and irregular or hexagonal plates.

They generally appear in the urine colored brownish red although occasionally they are colorless. The presence of uric acid in the urinary sediment does not necessarily indicate any pathologic condition; neither does it mean that the uric acid content of the urine is increased. The pathologic conditions in which uric acid is found in the sediment are as follows: gout, acute febrile conditions, and chronic nephritis.

Cystine crystals: These crystals are rarely found in urinary sediments but may produce concretions in the bladder. Cystine is insoluble in water, alcohol, and acetic acid and is soluble in hydrochloric acid, alkalies, and especially in ammonia. This solubility in ammonia distinguishes it from uric acid. Cystine contains considerable sulfur. Cystine occurs in all members of certain families instead of uric acid, and in such families it appears to replace uric acid; in them cystine calculi are not uncommon. Cystine consists of hexagonal colorless plates of moderate sizes and of high refraction which, in side view present 1 perfect facet and 2 imperfect neighboring facets. A number of plates may lie together, one upon an-

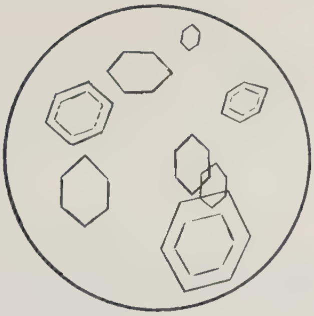

Fig. 642

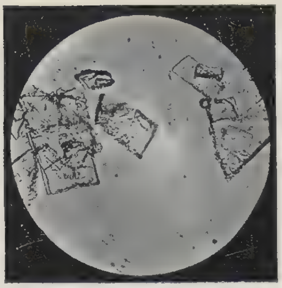

Fig. 643

Fig. 642. Cystine crystals.

Fig. 643. Cholesterol crystals. (×200.)

other, or they may form more or less regular masses.

Cholesterol crystals: These crystals are rarely found in urinary sediments. They ordinarily crystallize in regular and irregular colorless plates which are transparent. They may occasionally be found as a film on the surface of the urine instead of in the sediment. The pathologic conditions in which cholesterol crystals have been found in the urine are as follows: cystitis, pyelitis, chyluria, and nephritis.

Hippuric acid crystals: These crystals are rarely found in urinary sediments; they appear as needles or prisms which are generally pigmented like uric acid crystals. They are more soluble in water and ether than are uric acid crystals and have practically no clinical significance.

Leucine and tyrosine crystals: These crystals almost always appear in the urine together, in solution or as a sediment. Leucine crystallizes in characteristic spherical masses and is highly refractive. Tyrosine crystals appear as sheaves of very highly refractive needles.

The pathologic conditions in which leucine and tyrosine have been found are acute yellow atrophy of the liver, acute phosphorus poisoning, cirrhosis of the liver, severe cases of typhoid fever, severe cases of smallpox, and leukemia.

Urates

Urates may appear as ammonium, calcium, magnesium, potassium, and sodium urate. The calcium, magnesium, potassium, and sodium urates appear in acid urines, whereas the sediment of ammonium urate appears in neutral, alkaline, or acid urines.

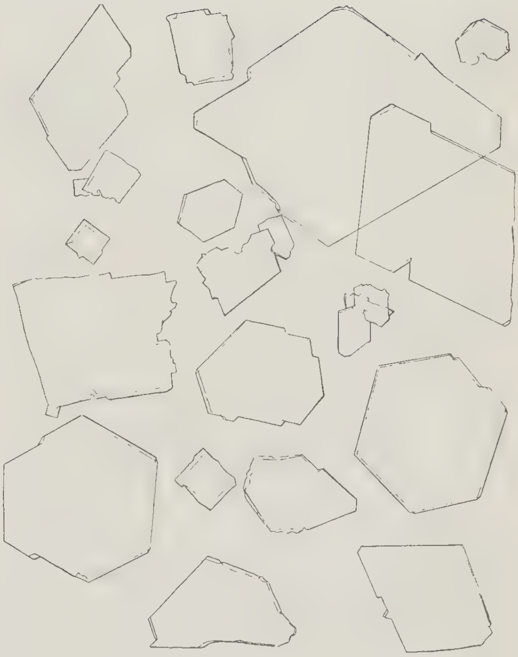

Fig. 644. Cholesterol crystals.

Sodium urate: Sodium urate may be amorphous or crystalline. When crystalline it appears in sheaves or clusters of colorless needles.

Ammonium urate: Ammonium urate generally appears in the burrlike form of the thornapple which appears to be balls with spicules attached. The pathologic conditions in which urates may appear in the urine are somewhat similar to those of uric acid.

Morphology of urinary crystals appearing during administration of sulfonamide drugs

The literature on the specific appearance of crystals produced during administration of sulfonamide drugs is somewhat scant. Lehr and

Text continued on p. 1865.

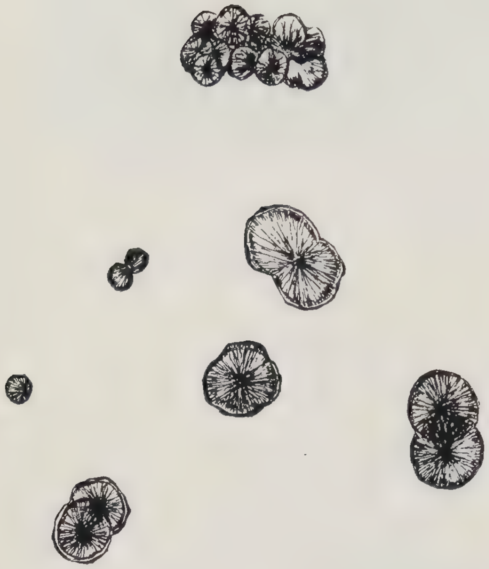

Fig. 645. Crystals of impure leucine. (After Ogden.)

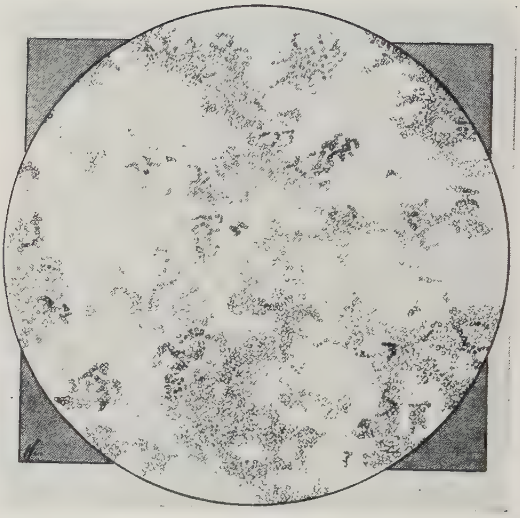

Fig. 646. Amorphous urates.

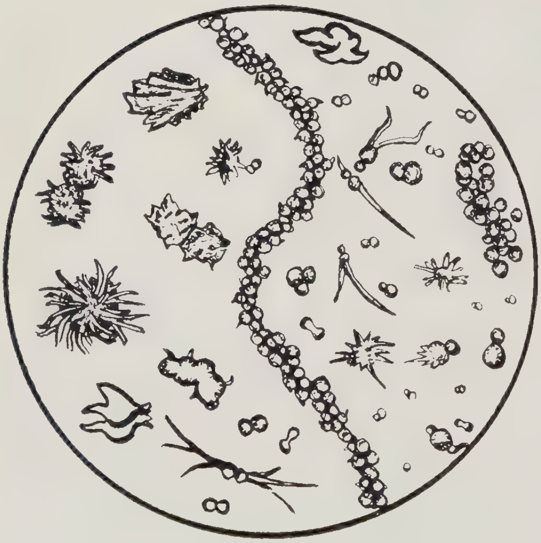

Fig. 647. Ammonium urate crystals. (After Peyer.)

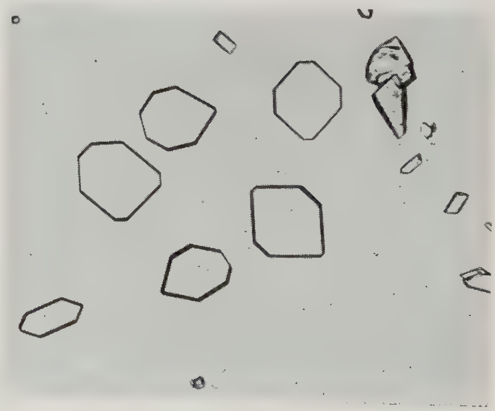

Fig. 648. Sulfanilamide crystals. (Courtesy Merck & Co., Inc., Rahway, N. J.)

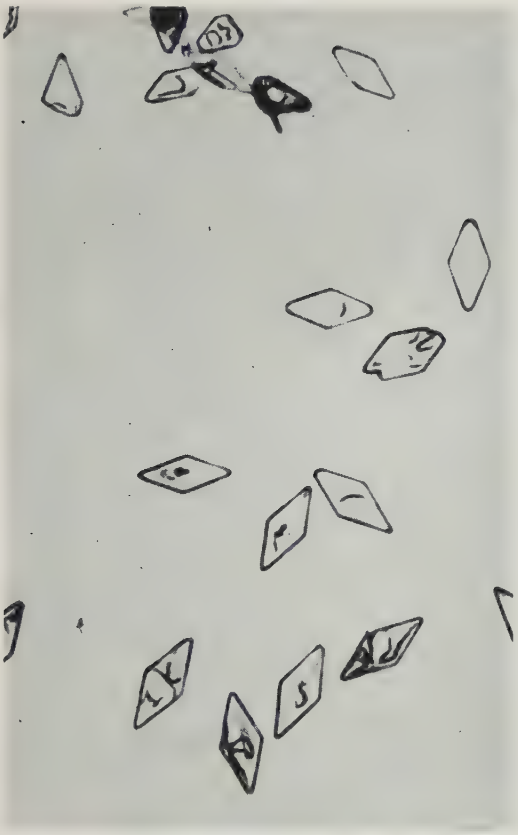

Fig. 649. Sulfapyridine crystals. (Courtesy Merck & Co., Inc., Rahway, N. J.)

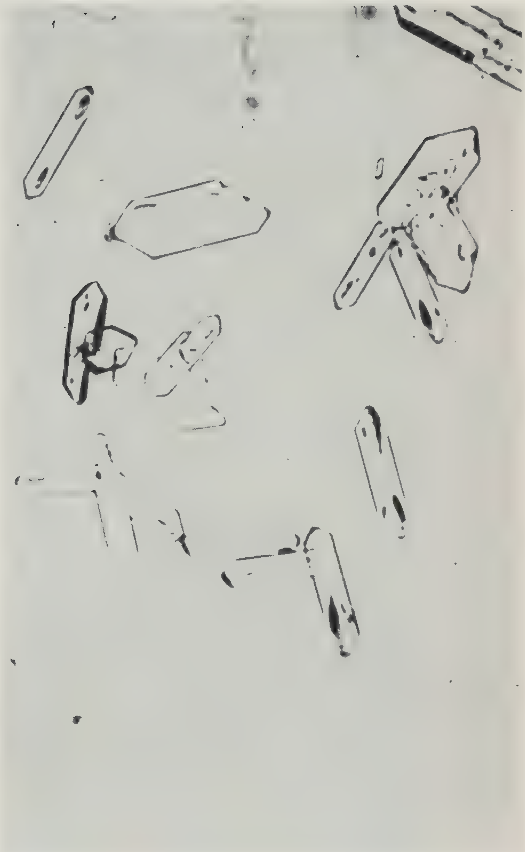

Fig. 650. Sulfathiazole crystals. (Courtesy Merck & Co., Inc., Rahway, N. J.)

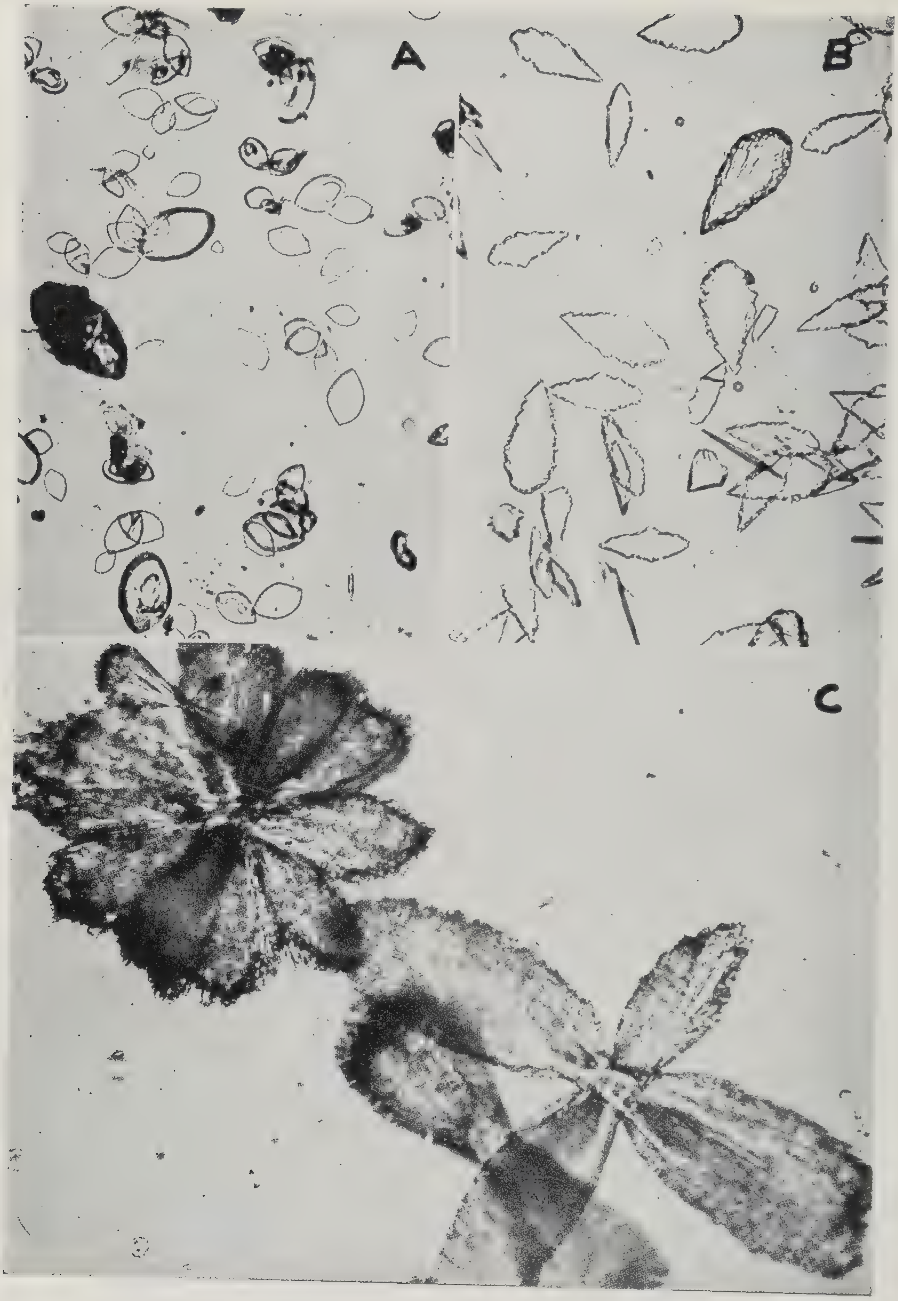

Fig. 651. Urinary crystals of acetylsulfapyridine. **A,** "Whetstones." Most common form; varying considerably in size (0.008 × 0.01 to 0.05 × 0.08 mm.), they are transparent, colorless, and usually show notching of serration of one or both edges. **B,** "Arrowheads" (0.016 × 0.56 to 0.04 × 0.92 mm.), similar to **A,** but wedge-shaped, presenting one sharp point and pronounced sawlike identification of one or both sides. The base is rounded and shows irregular notchings. **C,** Centrally waisted sheaves composed of needlelike crystals, usually rather large, as also pictured by Sadusk et al.; (largest crystal in the photograph 1 × 4 mm.). These sheaves may conglomerate and form crosslike and starlike structures. (×176.) (From Lehr, D., and Antopol, W.: Am. J. Clin. Path. **12:**4, 1942.)

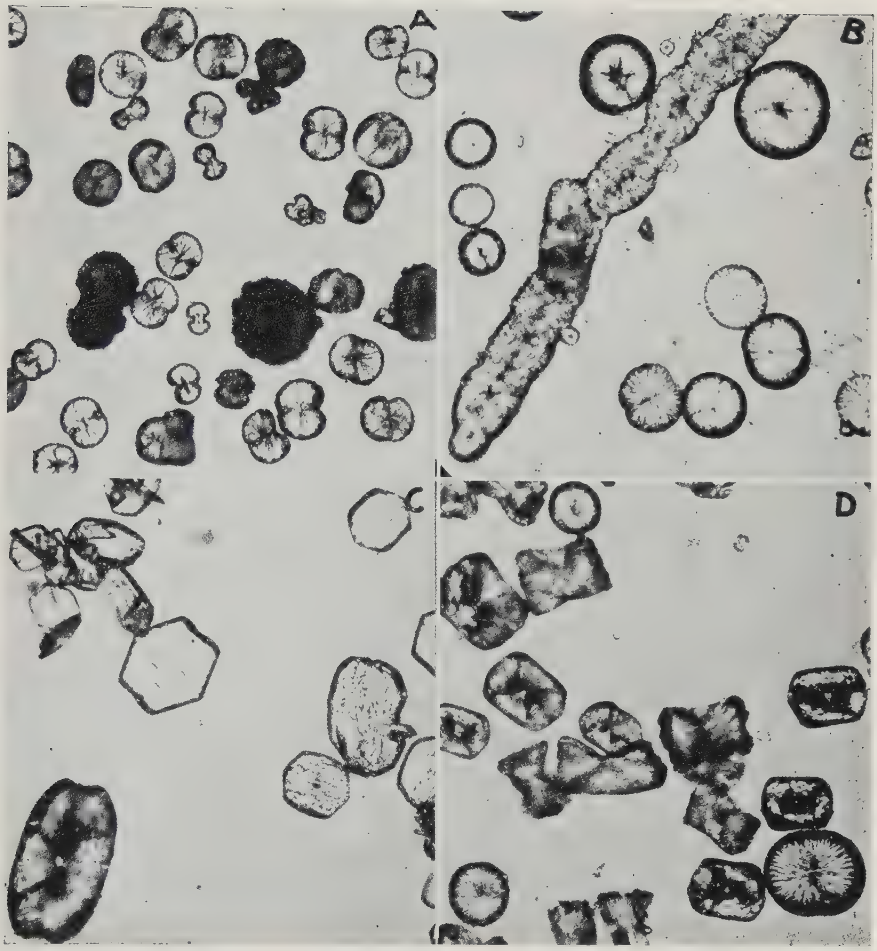

Fig. 652. Urinary crystals of acetylsulfathiazole. **A,** Dumbbells or "shocks of wheat with central binding"; the crystals start as small slender sheaves and grow in such a way that the 2 half-circle parts finally close up to form a rosette (0.004 × 0.06 to 0.06 × 0.08 mm.). **B,** Rosettes (0.04-0.1 mm.), the large rodlike structure consisting of acetylsulfathiazole, may represent a "crystalline cast." On microscopic examination both crystal forms (**A** and **B**) appear amber green and show pronounced radial striation. **C,** Hexagonal platelets (0.04 × 0.06 to 0.15 × 0.2 mm.). Sometimes very thin glass-clear, more often thick amber green with a distinct envelope-like pattern. Some of the crystals may reach considerable size and 2 opposite edges may appear indented resulting in shapes as shown in the right lower corner of the photograph. **D,** Simultaneous presence in the same urinary specimen of the 3 forms outlined in **A, B,** and **C,** a rather frequent observation. (×125.) (From Lehr, D., and Antopol, W.: Am. J. Clin. Path. **12**:4, 1942.)

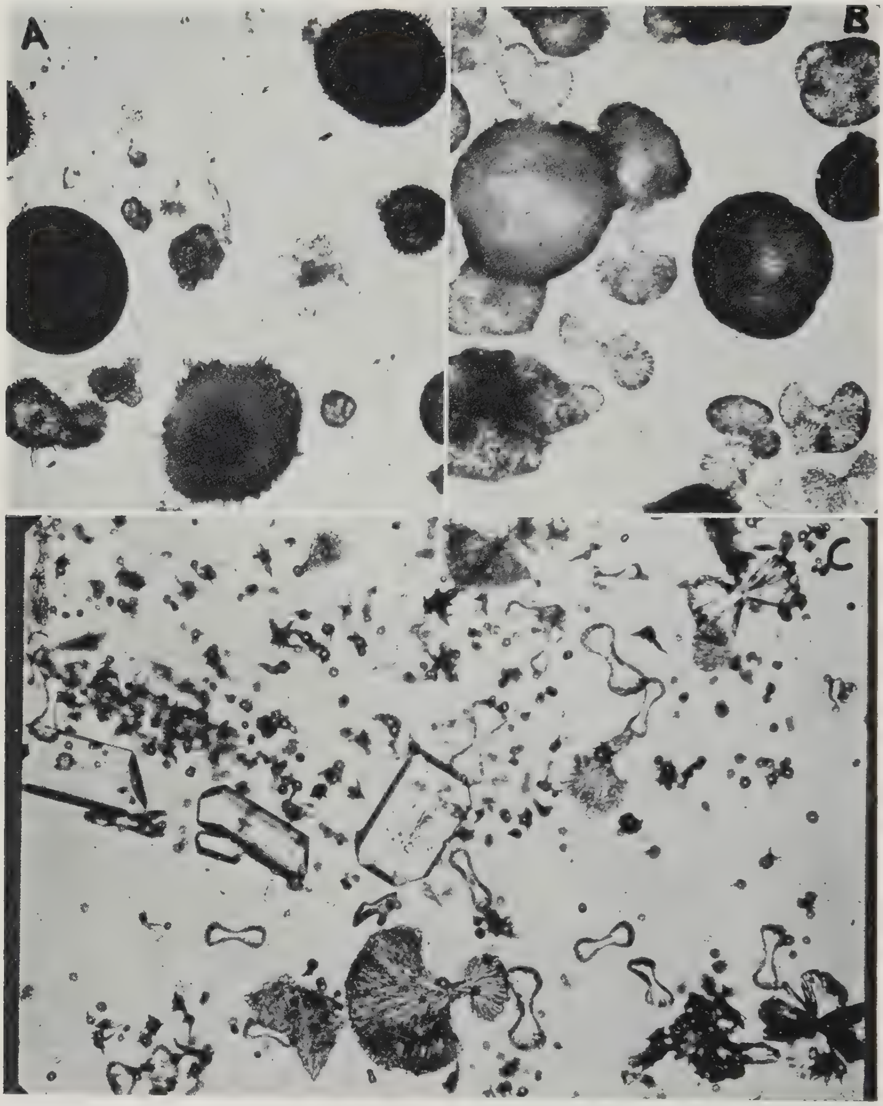

Fig. 653. Urinary crystals of sulfadiazine and acetylsulfadiazine. **A,** Dark, muddy green globules, sometimes covered with needlelike processes (chestnut burr forms), consisting almost entirely of free sulfadiazine (0.03-0.16 mm.). **B,** Among "globules" containing free sulfadiazine, "shocks of wheat with excentric binding" and "shell" forms, consisting mainly of acetylsulfadiazine. Like the corresponding sulfathiazole crystals they are amber green and have a conspicuous radial striation (0.02-0.14 mm.). **C,** Urinary sediment of a patient who had been switched from sulfathiazole to sulfadiazine treatment 24 hr. before voiding the urine specimen containing these crystals. A perfect "shock of wheat with excentric binding" (acetylsulfadiazine) can be seen among symmetrical dumbbells (acetylsulfathiazole). Also present are typical phosphate and monourate crystals. (×150.) (From Lehr, D., and Antopol, W.: Am. J. Clin. Path. **12:**4, 1942.)

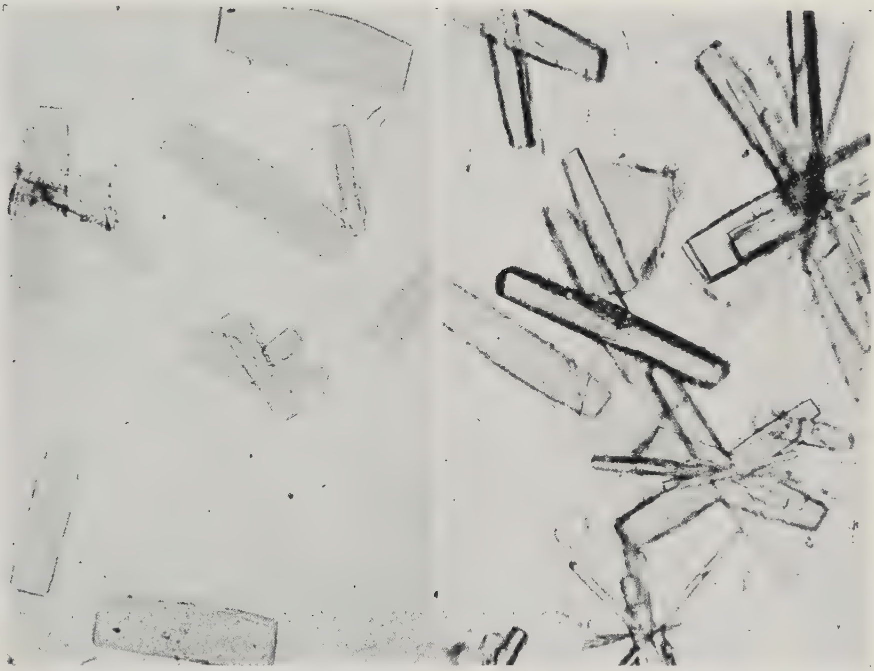

Fig. 654. Urinary crsytals of acetylsulfaguanidine. Simple rectangular oblong plates with slight bulging in the long axis. Markedly varying in size (0.02 × 0.09 to 0.05 × 0.24 mm.), these plates are either glass-clear or have a fine meshlike pattern, which makes them appear like isolated plant cells or slender tapeworm proglottides. They tend to conglomerate, forming crosslike or starlike structures. Repeatedly slender sheaves of needle crystals were seen interspersed among the plates (center in the upper part of the right photograph). (×138.) (From Lehr, D., and Antopol, W.: Am. J. Clin. Path. **12**:4, 1942.)

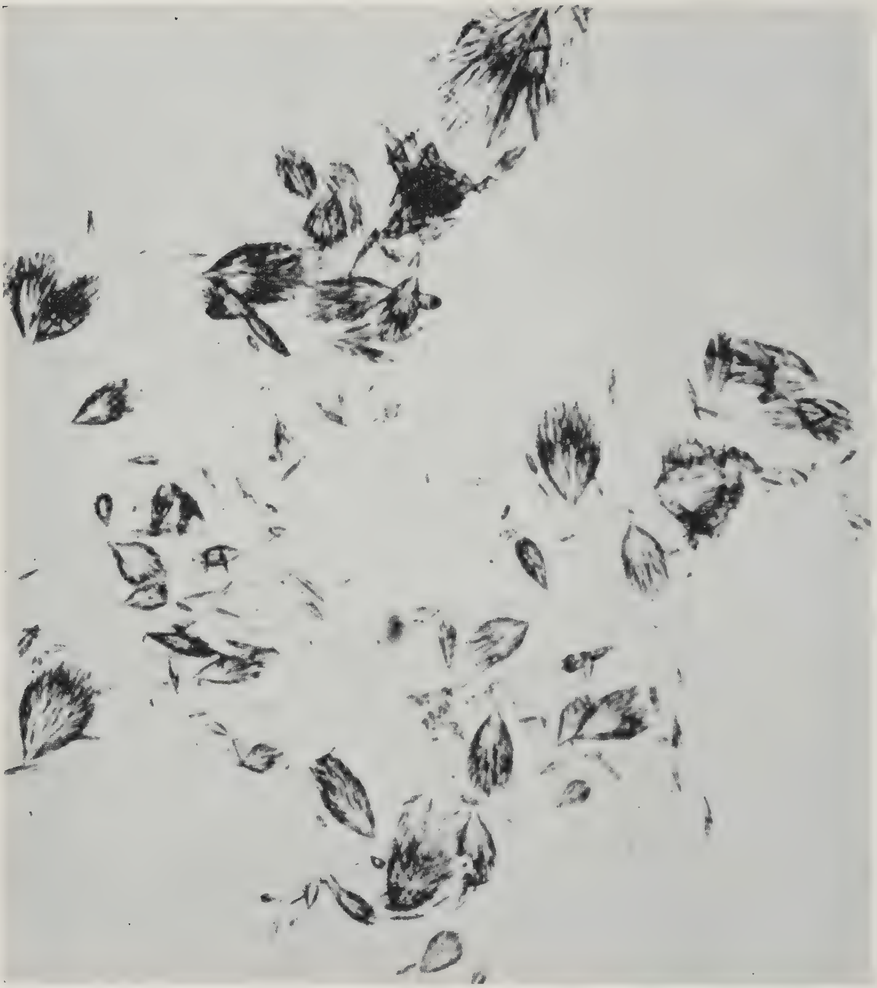

Fig. 655. Featherlike tufts of needle crystals, amber green, observed in urine of patient receiving sulfamethylthiazole. Unfortunately these crystals were not saved for analysis. The general discontinuance of sulfamethylthiazole therapy makes further investigations difficult. (×250.) (From Lehr, D., and Antopol, W.: Am. J. Clin. Path. **12**:4, 1942.)

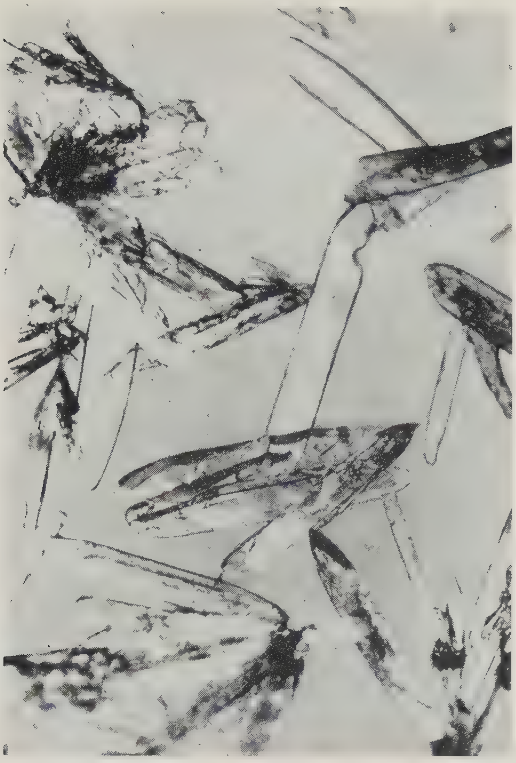

Fig. 656. Crystalline form of the sodium salt of penicillin G. (Courtesy Merck & Co., Inc., Rahway, N. J.)

Antopol[31] have made a study of this question on 260 individual urinary specimens from 175 patients to whom these various drugs have been administered. The drugs studied were sulfapyridine, sulfathiazole, sulfadiazine, and sulfaguanidine. They state that the frequency and quantity of these crystals in the urine are apparently related to the solubility of the sulfanilamide compounds and their acetylated compounds. The comparatively insoluble acetyl derivatives formed in the body from sulfapyridine, sulfathiazole, and sulfadiazine, if analyzed as urinary crystals, show a depressed melting point and a solubility which is about twice as high as that of the chemically acetylated drugs. This is true, too, of artificially produced urinary crystals. When one recrystallizes these substances from water, the shapes of these crystals and their melting points and solubility values approach those of the synthetic compounds.

The shape of urinary crystals derived from these drugs points to the influence of colloids upon crystal formation. These colloids appearing in the urine of patients taking these drugs may be due to damage to the renal filter apparatus. Lehr and Antopol state that the colloids may assume a protective action by inhibiting the precipitation of poorly soluble substances. The increase in solubility by a colloid-chemical mechanism would render additional explanation to the fact that the concentration of sulfapyridine, sulfathiazole, sulfadiazine, and their acetyl derivatives in the urine of patients treated with these compounds can significantly exceed the solubility of the same drugs in water and normal urine. This fact may be important in preventing precipitation or concrement formation in the urinary tract.

Lehr and Antopol showed photomicrographs (Figs. 651-655) which indicate the specific nature of the crystals of each of these compounds. They did not claim, however, that these pictures show **all** forms possible, nor did they deem it necessary to employ more photographs to show slight variations in the shapes described. They did state, however, that with the aid of these illustrations recognition of the particular drug has been possible on the first microscopic examination of urinary sediment in almost every case.

Chapter 102

Urinary calculi

SAM FRANKEL

Despite a vast amount of clinical observation and experimental research the problem of urinary lithiasis remains unsolved. Causation is believed to be linked with nutrition, although many exceptions exist. The problem of prevention of recurrence in the susceptible individual has been studied at length, and results have not been too successful.

Methods of analysis of urinary calculi have been devised to ascertain their exact composition with the end in view of utilizing this knowledge to prevent recurrence.

OPTICAL AND X-RAY CRYSTALLOGRAPHY

Because of the inadequacies of chemical methods of analysis as applied to identification of urinary calculi, mineralogic methods[32] have been applied, and these methods are presented here.

Urinary calculi can be divided into 2 general classes, crystalline, and amorphous. The term amorphous has been loosely applied to phosphatic concretions that are grossly opaque, dull, chalky, and apparently noncrystalline. With the exception of those exceedingly rare calculi that are composed of fibrin and bacteria it may be stated that all urinary calculi are crystalline. When the so-called amorphous calculi are broken up into a coarse powder and examined under a petrographic microscope, the fragments exhibit optical properties of crystalline substances and also give distinct and characteristic x-ray diffraction patterns.

Optical methods

Chemical compounds tend to form crystals when they pass from a liquid or gaseous state to the solid state. The external geometric form and internal molecular structure of such crystals are distinct, constant, and characteristic for any particular substance. Even when conditions in the environment are unfavorable for the development of visible geometric crystal faces, the formless crystalline mass has the same internal atomic structure.

Light passing through transparent crystalline material is influenced by the crystal structure according to the laws of optics, and these changes in transmitted light can be measured accurately. This results in data called **optical constants.** The polarizing or petrographic microscope is a useful instrument for measuring external and internal physical properties of crystalline material. A series of compounds of known chemical composition is analyzed for optical constants, and unknown substances are recognized by measuring their optical constants and comparing with prepared tables.

Another method is the so-called immersion method in which a minute quantity of coarsely ground calculus is placed upon a glass slide and immersed in a succession of liquids of known indices of refraction. A match is found for the indices of refraction. Other optical constants, including optical sign, axial angle, dispersion, extinction angle, sign of elongation, etc., may be measured at the same time. A milligram of substance is usually required, but measurements can be made on a few microscopic grains by transferring them from slide to slide.

Constituents of urinary calculi are relatively few and markedly different in optical behavior, which makes the problem of identification simple. Only a few diagnostic optical tests need usually be applied to identify a

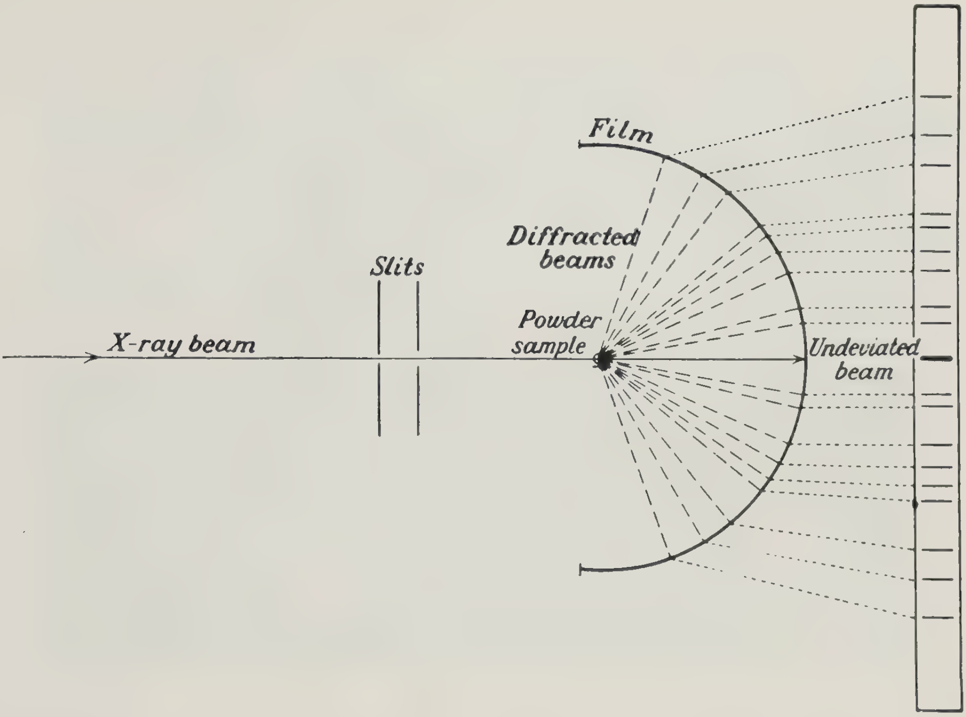

Fig. 657. Diagram showing method of obtaining x-ray powder photographs. (From Prien, E., and Frondel, C:. J. Urol. 57:949, 1947.)

substance. Confirmatory tests can be made at times.

Analysis by x-ray methods—Hull or Debye-Scherrer ("powder" method)

The method consists in irradiating a finely powdered sample of a substance with a beam of monochromatic x-rays and recording the diffracted (reflected) rays in a suitably arranged camera. The rays are reflected from planes of atoms in the substance, to be recorded as a series of lines upon a circular photographic film, forming a characteristic pattern for the substances irradiated. A diagram of the method is seen in Fig. 657. Two methods of identification may be used:

1. Spacings of the lines on the films are measured and the interplanar spacings are calculated for the substance in question. Comparison is made with a tabulated list of interplanar spacings of known chemical substances.*

2. Comparison is made of the unknown patterns with standard x-ray patterns of known substances.

For this method of testing, the size of the sample may be quite small, 1 mg., but 0.1 mg. or even less will suffice. The sample may be first examined optically, then ground to a fine powder and examined by x-ray. It is possible with this method to examine mixtures and to identify the several constituents and to estimate the approximate proportions of each.

Spacing data on x-ray powder diffraction may be obtained in the original article.[32]

TECHNIC OF OPTICAL ANALYSIS

Examination with ordinary light

Equipment:

1. Stereoscopic binocular dissecting microscope.
2. Lamp.
3. Biologic dissection needles (a small machinist's vise fitted with a heavy needle is excellent.
4. Small mortar and pestle for powdering calculi.
5. Watch glasses to hold powdered samples.
6. Corked small glass vials or bottles.
7. Pair of cutting pliers or dental extraction forceps for breaking large or firm stones.

Procedure:

1. Transfer samples of powder to the blade of a small knife. Use watch-maker's forceps or tweezers to transfer individual grains or crystals that have been spotted in a sample under magnification.
2. Fracture of the calculus permits inspection of such structural characteristics as texture, lamination, porosity, and the study of in-

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*This list is procurable from Dow Chemical Co. or from the American Society for Testing Materials.

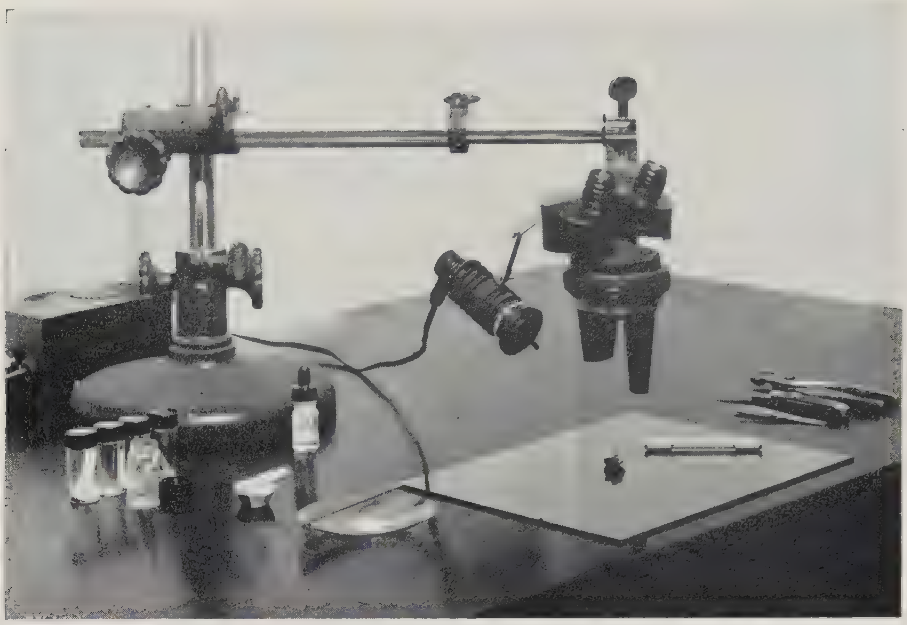

Fig. 658. Equipment for examination of calculus with ordinary light. Includes binocular stereoscopic microscope, lamp (mounted on microscope here), pliers to fracture hard calculi, mortar and pestle to grind calculi, knife blade or spatula to transfer powder, watch glasses to hold powder, pointed tool for picking at laminations, nucleus, etc., stoppered bottles for filing small calculi and powder. (From Prien, E., and Frondel, C.: J. Urol. **57**:949, 1947.)

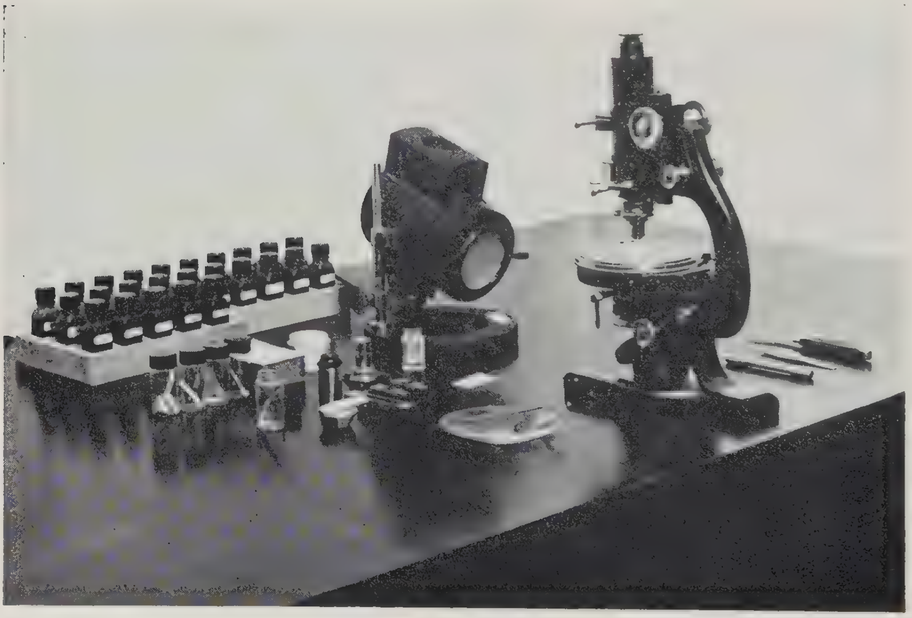

Fig. 659. Equipment for examination of calculus with polarized light. Includes petrographic microscope, lamp, set of immersion liquids with graduated indices of refraction from 1.41 to 1.75 (in bottles in rack), glass slides and cover slips, also implements for examination with ordinary light. (From Prien, E., and Frondel, C.: J. Urol. **57**:949, 1947.)

dividual crystals and is necessary if the nucleus is to be observed. The calculus should be thoroughly dried before analysis is attempted.
(a) Fracture the calculus.
(b) Inspect external and fractured surfaces with the low magnification (10× or 20×) of a hand lens or binocular stereoscopic microscopic (dissecting microscope) (Fig. 658).

Examination with polarized light

1. Remove minute samples with pointed tool and grind to a coarse powder in a mortar with pestle.
2. Place powder upon a glass slide, cover with liquid of known index of refraction, and place a glass cover slip over the specimen. Examine upon the stage of a polarizing microscope (Fig. 659).

Immersion liquids have indices 1.41-1.75, graduated in steps of at least 0.01. Liquids of intermediate index, approximately accurate to 0.005, may be prepared by mixing equal droplets of liquids of 2 adjacent steps in the series. This may be done on the glass slide but is not necessary in routine analysis. Accuracy to 0.01 is sufficient for the differentiation of all the substances in Table 364.

To analyze calculi composed of several components it is possible to approximate the percentage of each component by powdering the entire calculus, stirring the fragments thoroughly to obtain a uniform mixture, and then making grain counts on random samples through the binocular and petrographic microscopes. It is possible to estimate the percentage composition to within 5 or 10%, which is sufficient for most purposes.

CRYSTALLINE COMPONENTS OF URINARY CALCULI

In his studies of urinary calculi, Prien et al. found that pure calcium oxalate calculi constituted 36.1% of the total; mixed calcium oxalate-apatite calculi comprised 31%; or 67.1% of the total were included in this group. These calculi usually but not always occur in acid sterile urine.

Pure magnesium ammonium phosphate hexahydrate, pure apatite, and mixed magnesium ammonium phosphate hexahydrate-apatite calculi comprised 19.5% of the total. These calculi usually but not always occur in alkaline infected urine.

Calcium hydrogen phosphate dihydrate occurred in 1.6% of the calculi.

Uric acid and cystine exist more frequently in pure than in mixed form and occurred in 6.1% and 3.8%, respectively, of the series.

Sodium acid urate occurred but once in the series and in microscopic amount. It was the only urate found.

In a study of approximately 700 urinary calculi only 9 distinct crystalline substances were found. These are divided into the following groups: oxalates, phosphates, uric acid, urates, and cystine.

Calcium oxalate

Calcium oxalate is the most frequent constituent found in urinary calculi.

Calcium oxalate monohydrate (whewellite—$CaC_2O_4 \cdot H_2O$): This is more common than the dihydrate. The simplest form is the small smooth ovoid "hempseed" stone, usually multiple, and consists of concentric laminations, Fig. 660, *1* and *2*.

The second type is the "mulberry" stone of variable size and shape with irregular rounded mammillary processes (Fig. 660, *3* and *4*). On cross section these rounded protuberances are seen to be composed of radially striated aggregates. These radiating fibers are much finer than those of another fibrous constituent of calculi, $CaHPO_4 \cdot 2H_2O$, and may be almost microscopic (Fig. 661, *1*). Some predominately fibrous (radially bladed) calculi may have a finely or coarsely granular nucleus of the same material (Fig. 661, *1*).

The third type of calculus is the uncommon "jackstone" which is usually found in the

Table 363. *Formula and minerologic name of urinary calculi*

Found	*Formula*	*Mineralogic name*
Calcium oxalate monohydrate	$CaC_2O_4 \cdot H_2O$	Whewellite
Calcium oxalate dihydrate	$CaC_2O_4 \cdot 2H_2O$	Weddellite
Magnesium ammonium phosphate hexahydrate	$MgNH_4PO_4 \cdot 6H_2O$	Struvite
Carbonate-apatite	$Ca_{10}(PO_4 \cdot CO_3OH)_6(OH)_2$	Carbonate-apatite
Hydroxyl-apatite	$Ca_{10}(PO_4)_6(OH)_2$	Hydroxyl-apatite
Calcium hydrogen phosphate dihydrate	$CaHPO_4 \cdot 2H_2O$	Brushite
Uric acid	$C_5H_4N_4O_3$	
Cystine	$SCH_2CH(NH_2)—COOH$	
Sodium acid urate (not found but identity established)	$NaH \cdot C_5H_2O_3N_4 \cdot H_2O$	
Tricalcium phosphate (not found but identity acceptable)	$Ca_3(PO_4)_2$	Whitlockite
Indigo	$C_{16}H_{10}N_2O_2$	
Xanthine	$C_5H_4N_4O_2$	

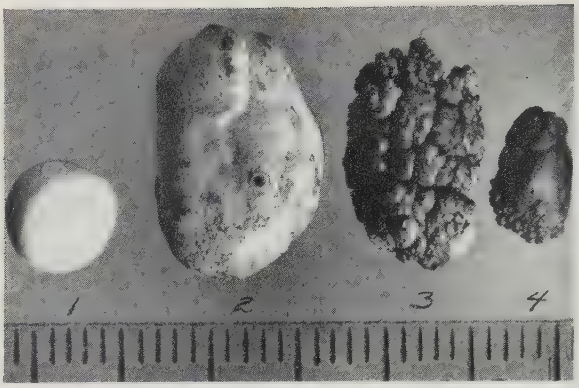

Fig. 660. Calcium oxalate monohydrate ($CaC_2O_4 \cdot H_2O$) calculi. **1** and **2**, Calculi of "hemp-seed" variety, small, smooth. **3** and **4**, Calculi of "mulberry" variety, externally showing rounded mammillary surfaces. Scale in millimeters. (From Prien, E., and Frondel, C.: J. Urol. **57**:949, 1947.)

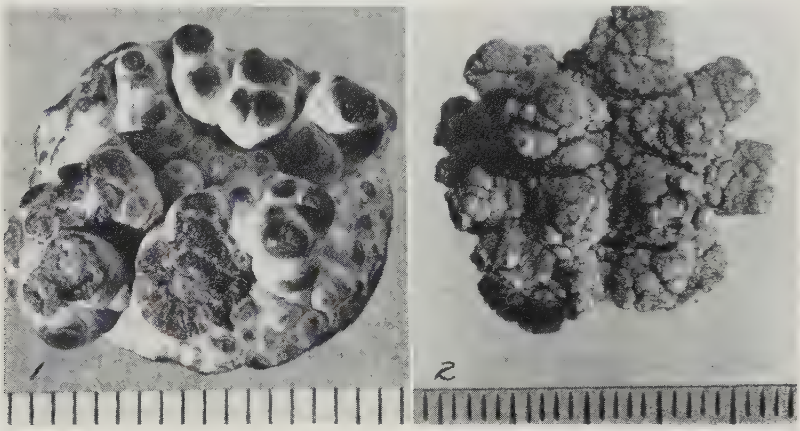

Fig. 661. 1, Calcium oxalate monohydrate calculus. Typical appearance of "mulberry" stone. Fracture of mammillary process shows concentric lamination and radial striation with central granular nucleus. **2,** Calcium oxalate "jackstone," a central mass of calcium oxalate monohydrate with radiating blunt spinous processes. A fine deposit of calcium oxalate dihydrate crystals is precipitated over the exterior. Scale in millimeters. (From Prien, E., and Frondel, C.: J. Urol. **57**:949, 1947.)

bladder and consists of a dense central mass with radiating spicules (Fig. 661, *2*).

Calculi composed of calcium oxalate monohydrate are occasionally porous or cavernous, especially toward the interior; or a particular layer in a laminated stone may be porous. The granular type of structure occurs most commonly in those calculi of mixed composition. Both the fibrous and granular calculi may show growth banding (lamination) marked by color differences parallel to the surface of the stone. Calcium oxalate monohydrate usually forms calculi that are extremely dense, tough, and hard, and it is usually the substance found when it is reported that a calculus resisted crushing or sawing.

External surface—usually smooth and may have a lustrous or varnished appearance.

External color—light honey brown to red brown or black brown or occasionally blue to greenish blue.

Fractured surface—usually some shade of brown.

Finely granulated calculi—relatively light colored.

Nuclei of other substances, uric acid and apatite, are sometimes observed, and calcium oxalate monohydrate may occur as nuclei in uric acid and other stones. Calcium oxalate dihydrate is the most common associate of calcium oxalate monohydrate and usually occurs as well-formed crystals attached to or embedded in the surface of the monohydrate stone. Apatite also is a common associate; magnesium ammonium phosphate hexahydrate (struvite), calcium hydrogen phosphate dihydrate (brushite), and uric acid are infrequent.

Calcium oxalate monohydrate is monoclinic in crystallization and possesses cleavages on (001), (010), and (110). Cleavages can be recognized in relatively large single crystals but are not easily seen in fibrous or fine-granular aggregates.

Hardness 2½-3
Specific gravity 2.23
Optical properties:
  $X = 1.491 = b$
  $Y = 1.555$
  $Z = 1.650$
  Biaxial positive (+)
  $2V = 84°$
  Dispersion $r < v$, weak
  Extinction angle $Z \wedge c = 31°$

It is easily distinguished optically from calcium oxalate dihydrate, $CaC_2O_4 \cdot 2H_2O$ by its high birefringence and high Z index of refraction.

Occasionally the granular type of calculus may show well-defined tiny crystals upon its surface (Fig. 662, *1, 2, 3*).

Calcium oxalate dihydrate (weddellite—$CaC_2O_4 \cdot 2H_2O$): This generally occurs as relatively well-formed crystals, shaped as in Fig. 662, *4,* which range in size up to 2 mm. on an edge. They are identical with the familiar octahedral or "envelope" crystals seen in ordinary urinary sediments. The crystals are usually attached to the body of the stone by their edges. Calculi composed of pure calcium oxalate dihydrate consist either of loosely intergrown aggregates of individual crystals (Fig. 662, *1*) or of compact, interlocking aggregates with a coarsely crystallized surface (Fig. 662, *2*). A secondary crop of smaller crystals may be superdeposited. In calculi of mixed internal composition a granular structure may exist, with no development of crystals. No nucleus is found in most of the loosely aggregated calculi. Pure dihydrate calculi are much less common than are pure monohydrate stones.

The color of the dihydrate is pale yellowish white to honey brown, and it is lighter than the monohydrate. The two hydrates are frequently associated; the dihydrate usually occurs as a surface incrustation of well-formed crystals upon or slightly embedded in the monohydrate. The contrast between the 2 forms may be quite striking (Fig. 662, *4*). The dihydrate is usually of later formation than the monohydrate but in part has overlapped the closing stages of growth of the monohydrate. Much less commonly the 2 may be intimately associated in those granular oxalate calculi which lack any regularity of structure. Apatite is less commonly associated with the dihydrate. Frequently the 2 oxalates and apatite exist together. Infrequent associates are with calcium hydrogen phosphate dihydrate (brushite), cystine, and uric acid.

Prien believes that both the monohydrate and the dihydrate are direct products of crystallization from the urine.

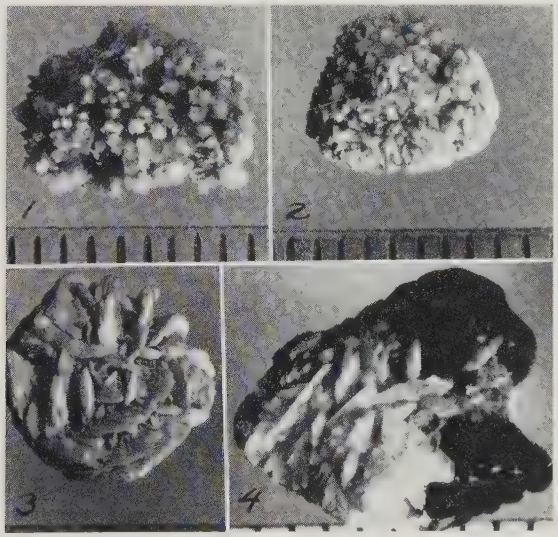

Fig. 662. Calcium oxalate dihydrate ($CaC_2O_4 \cdot 2H_2O$) calculi. **1,** Loose aggregate of crystals. **2,** Compact aggregate of crystals. **3,** Unusually large crystals seen edgewise. **4,** Dihydrate crystals (white) deposited upon calcium oxalate monohydrate calculus (black). Knifelike edges of dihydrate crystals are well shown. Scale in millimeters. (From Prien, E., and Frondel, C.: J. Urol. **57:** 949, 1947.)

Calcium oxalate dihydrate is tetragonal in crystallization. It has no cleavage.

Hardness 4
Specific gravity 1.99
Optical properties:
  O = 1.523
  E = 1.544
  Uniaxial positive (+)

It is distinguished optically from calcium oxalate monohydrate by its much lower birefringence and lower indices of refraction. Microscopically the crystals commonly show growth banding (lamination) parallel to the crystal faces.

Optical data are lacking for the trihydrate. Oxalates other than the dihydrate and the monohydrate have not been observed in urinary calculi by Prien. Prien has found stone-forming patients who produce pure monohydrate or dihydrate calculi as well as some who produce concretions composed of a mixture of the 2 hydrates. The urines of patients who produce dihydrate calculi may contain fused masses (gravel) of the familiar "envelope" crystals. It appears that calculi may at times be formed by the normal crystalline salts in urine, contrary to statements in the literature.

Phosphates

Phosphates in urinary calculi present a complex problem which has not previously been resolved by chemical methods of study. Four different phosphates have been found. Two of these, magnesium ammonium phosphate hexahydrate (struvite) and apatite, form the great bulk of the material. A third, calcium hydrogen phosphate dihydrate (brushite), is relatively uncommon, being found but 12 times in 700 calculi. The fourth, tricalcium phosphate (whitlockite), has been reported in several instances. Magnesium ammonium phosphate, triple phosphate, is usually identified in the chemical analysis of a phosphatic stone. Commonly reported with it are calcium phosphate and calcium carbonate; these separate substances have not been found in this study but are combined in one complex compound, carbonate-apatite, a carbonated calcium phosphate.

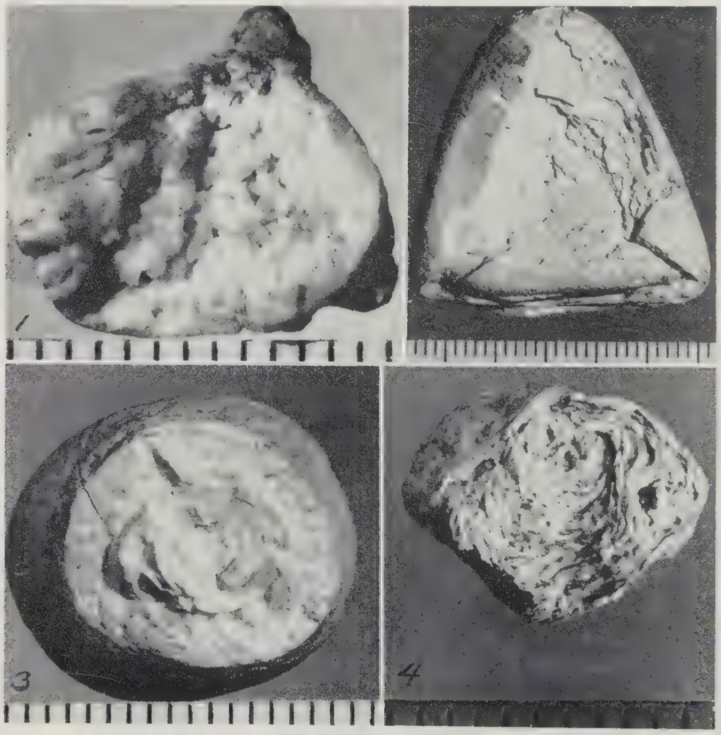

Fig. 663. Phosphatic calculi. **1,** Practically pure $MgNH_4PO_4 \cdot 6H_2O$, "triple phosphate," showing developing columnar structure. **2-4,** Pure apatite calculi. **2,** Flaking of laminations from dry calculus (triangular outline due to molding by bladder, 4 similar stones being present). **3,** Three types of apatite structure shown, central loose fluffy nucleus, intermediate zone of very dense laminations, and peripheral zone of less dense laminations. **4,** Alternating light and dark laminations are all pure apatite. Scale in millimeters. (From Prien, E., and Frondel, C.: J. Urol. 57:949, 1947.

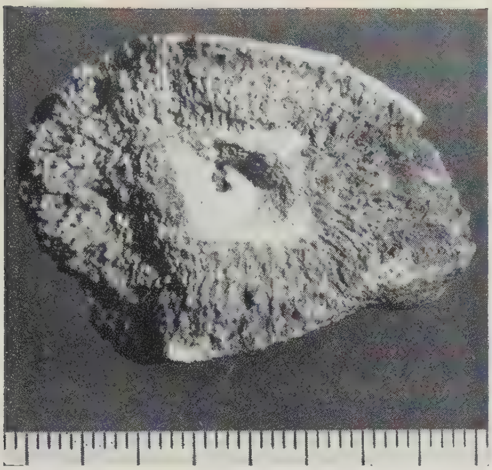

Fig. 664. Fractured surface of "staghorn" calculus. Central nucleus of apatite is surrounded by columnar structure of $MgNH_4PO_4 \cdot 6H_2O$, "triple phosphate." Intercolumnar spaces are filled with apatite. Scale in millimeters. (From Prien, E., and Frondel, C.: J. Urol. 57:949, 1947.)

Magnesium ammonium phosphate hexahydrate (Struvite—$MgNH_4PO_4 \cdot 6H_2O$ [triple phosphate]): This is usually associated with apatite in calculi. Pure calculi composed of it are quite uncommon, producing a densely granular creamy white appearance somewhat resembling lump sugar (Fig. 663, *1*). A suggestion of columnar structure is frequently present. In association with apatite, this substance forms the so-called "staghorn" calculi which occur in alkaline infected urine.

Its color is dirty to creamy white; the structure, typically cellular or columnar (Fig. 664), often resembles cancellous bone.

Triple phosphate and apatite crystals exist together in all degrees of admixture. A study showed that approximately half were composed predominantly of the one substance and half were composed of the other.

Well-formed single crystals of magnesium ammonium phosphate hexahydrate may be observed in open cracks in banded or laminated calculi of apatite and also projecting into cavities in porous stones. Crystals may also be found embedded in the central portions of some calculi where the apatite may be loose, fluffy, and grossly structureless. They also occur in urinary sediments as the well-known "knife-rest" or "coffin-lid" crystals. Crystals of typical shapes are shown in Fig. 673, *6, 7, 8*. They lack a center of symmetry in that the upper and lower halves are not mirror images.

Calcium oxalate monohydrate is an uncommon associate of magnesium ammonium phosphate hexahydrate, and the dihydrate is rare. One calculus was observed to have a nucleus of calcium oxalate monohydrate, an intermediate layer of calcium hydrogen phosphate dihydrate, and a periphery of magnesium ammonium phosphate hexahydrate. Uric acid was also observed as the nucleus of a calculus composed of magnesium ammonium phosphate hexahydrate and apatite.

It is orthorhombic-hemimorphic in crystallization. Crystals have a nearly perfect cleavage on (001) and an indistinct cleavage on (010).

Hardness 2
Specific gravity 1.71
Optical properties:
- $X = 1.495 = a$
- $Y = 1.496 = c$
- $Z = 1.504 = b$
- Biaxial positive (+)
- $2V = 37°$
- Dispersion $r < v$, strong

Microscopically it is easily distinguished by its very low birefringence and low indices of refraction.

Apatite (carbonate-apatite — $Ca_{10}(PO_4 \cdot CO_3OH)_6(OH)_2$; hydroxyl-apatite — $Ca_{10}(PO_4)_6(OH)_2$): The term apatite refers to a group of compounds that have an analogous chemical composition and identical crystal structure. They are mostly minerals. Carbonate-apatite and hydroxyl-apatite are complex phosphates occurring in calculi and belong to this group.

Calcium hydrogen phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$) uncommonly exists in calculi, and tricalcium phosphate ($Ca_3(PO_4)_2$) occurs rarely. Prien has not found calcium carbonate ($CaCO_3$) in apatite calculi.

Carbonate-apatite and hydroxyl-apatite can be identified as apatite only by optical or x-ray methods. They are then differentiated from each other by releasing carbon dioxide gas from carbonate-apatite. If a large amount of gas is evolved from an apatite substance occurring in calculi, it is inferred to be wholly or preponderantly carbonate-apatite; if little or no gas is evolved, it seems to be wholly or preponderantly hydroxyl-apatite. They should be considered collectively and called urinary apatite to distinguish them from mineral apatite, which usually contains chlorine or fluorine ions.

Apatite commonly occurs with magnesium ammonium phosphate hexahydrate as one of the major components of phosphatic calculi. It is by far the most common phosphate present in calculi. Pure apatite calculi are not common; they are fine-grained, soft, and compact, usually with concentric lamination, both on macroscopic and microscopic scale (Fig. 663, *2, 3, 4*). Successive laminae may vary somewhat in color and coherency, and some laminae may be relatively hard with a translucent glassy appearance. Inner portions of some stones are loose and powdery (Fig. 664).

The color of apatite is chalky white, cream white, yellow brown, or sometimes brown. Pure apatite is an alternating lamination of white and brown (Fig. 663, *4*) in which the brown layers represent a condensation of the substance. Brown layers may be translucent or even transparent.

In mixtures with magnesium ammonium phosphate hexahydrate in "staghorn" calculi the apatite is softer and may usually be rubbed to a fine powder with the finger. In porous granular calculi composed of the oxalates it exists in interstices or cavities in variable amounts. Commonly the interspaces in the loose aggregates of calcium oxalate dihydrate crystals may be partially or completely filled with the substance; Fig. 665 shows such aggregates. It has also been observed with calcium hydrogen phosphate dihydrate, cystine, and uric acid. Calculi of apatite and magnesium ammonium phosphate hexahydrate commonly occur in alkaline infected urines.

It is hexagonal in crystallization, well-formed crystals being found only in nature. Hardness—5; specific gravity—2.95-310. Natural crystals are optically negative, with O and E indices in the range from 1.61 to 1.64, depending on the kind and amount of isomorphous substitution. In urinary calculi it appears as extremely fine-grained masses, never as visible crystals, and is isotropic. This character is due to the submicroscopic size of the crystals composing the mass with resultant aggregate polarization. Index of refraction—1.55-1.61, most samples falling between 1.55 and 1.59. This variable and low index is partly due to a large content of adsorbed and capillary water. Index may vary between different parts of the same sample. Some grains may show weak double refraction. Some grains ordinarily are colorless but may be pale yellow, brown, reddish brown, or greenish yellow due to foreign pigmenting matter.

It differs from other components of calculi in its isotropic or nearly isotropic character and the absence of crystal outlines. It may be confused with colloidal organic material when seen through the petrographic microscope because both are isotropic. The colloid frequently appears as a coating on crystals from which it may be differentiated readily. It is very confusing if on apatite, which does not form crystals. **Test for CO_2** which may be liberated from carbonate-apatite.

1. Place a few coarse grains on a glass slide and cover with a cover glass.
2. Run a drop of dilute hydrochloric acid under the edge of the cover glass.
3. If carbonate-apatite is present a brisk effervescence ensues which may be watched through low magnification.
4. To confirm that this is CO_2 add a drop of 10% barium hydroxide under the cover glass. A white cloud of barium carbonate forms about the stone, showing presence of CO_2. Failure to produce effervescence in material having the constants of carbonate-apatite indicates the presence of hydroxyl-apatite.

Colloidal apatite calculus is undoubtedly ordinary carbonate-apatite. "$Ca_3(PO_4)_2$" prostatic calculi of Huggins and Bear appear to be composed of carbonate-apatite. The powder

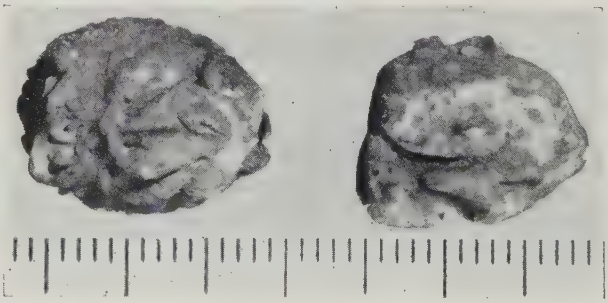

Fig. 665. Apatite upon calcium oxalate dihydrate. Linear edges of calcium oxalate crystals are seen upon the left, projecting through the apatite. On the right, calcium oxalate has been almost completely "submerged" by apatite, only the tips of the crystals being visible as irregularities in the surface. Scale in millimeters. (From Prien, E., and Frondel, C.: J. Urol. 57:949, 1947.)

pattern is actually that of hydroxyl-apatite. Although the percentage difference in composition between the 2 substances is very little, their atomic structures are quite unlike.

Carbonate-apatite is the principal constituent of calcifications variously formed in the lacrimal duct, prostate gland, tuberculous lymph nodes, appendix, testes, and walls of the bronchi as a consequence of senile degeneration and disease. It also occurs as a minor constituent in arteriosclerotic plaques.

A calcium phosphate kidney stone that afforded small amounts of CO_2 and H_2S when dissolved in acid was found to be carbonate-apatite containing a small amount of divalent sulfur in isomorphous substitution and may be termed a sulfidic apatite.

Tricalcium phosphate (whitlockite—$Ca_3(PO_4)_2$): Whitlockite is rhombohedral in crystallization. No cleavage.

Hardness 5
Specific gravity 3.2
Optical properties:
  O = 1.629
  E = 1.626
  Uniaxial negative (−)

Prien has identified whitlockite in a testicular calcification. It was creamy white, very fine-grained, and almost porcellanous in appearance, and contained a small amount of CO_2. It appeared isotropic, due to the very small particle size of the aggregate, and had a variable index of 1.59-1.60. Some of the natural material has also been found to contain CO_2 in isomorphous substitution analogous to carbonate-apatite. Urinary whitlockite probably has the same general appearance and optical properties as apatite. Probably the "$Ca_3(PO_4)_2$" reported in older descriptions of urinary calculi was apatite in most instances.

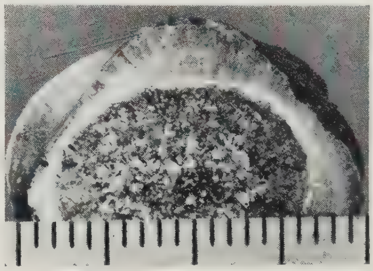

Fig. 666. Multilayered calculus. Nucleus of granular calcium oxalate monohydrate, second layer (almost invisible) of calcium oxalate dihydrate, third layer (broad) of laminated calcium hydrogen phosphate dihydrate, peripheral layer (very thin) of calcium oxalate dihydrate. Scale in millimeters. (From Prien, E., and Frondel, C.: J. Urol. 57:949, 1947.)

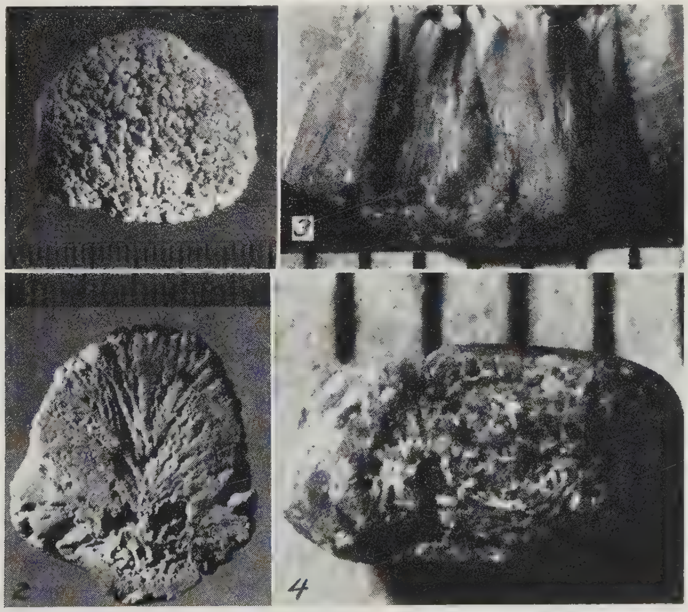

Fig. 667. Calcium hydrogen phosphate dihydrate ($CaHPO_4 \cdot 2H_2O$) in calculi. **1,** External surface of rare pure $CaHPO_4 \cdot 2H_2O$ calculus, showing ends of tufts of crystals, producing nodular structure. **2,** Cross section of same calculus showing divergent tufted structure. **3,** "Palisade" formation of $CaHPO_4 \cdot 2H_2O$, due to cleavage along fibers which are well shown. **4,** Bladed crystals of $CaHPO_4 \cdot 2H_2O$ in a granular calcium oxalate monohydrate calculus. Scale in millimeters. (From Prien, E., and Frondel, C.: J. Urol. 57:949, 1947.)

Calcium hydrogen phosphate dihydrate (brushite—$CaHPO_4 \cdot 2H_2O$): This is an uncommon phosphate occurring in urinary calculi, having been found but 12 times in 700 calculi. In at least 3 instances the calculi were present as bladder stones accompanying benign hypertrophy of the prostate and with urinary infection. Because it is an acid phosphate and is usually associated with the oxalates in calculi it is reasonable to suppose that it forms in an acid urine. It was accompanied several times by carbonate apatite, which also occurs commonly with the oxalates. In the single instance in which it occurred with magnesium ammonium phosphate hexahydrate the latter was present as a peripheral layer which could be explained as the result of a change in the reaction of the urine to the alkaline side subsequent to the precipitation of the calcium hydrogen phosphate dihydrate.

It may exist as a layer overlying a nucleus of calcium oxalate monohydrate or as bladed crystals in granular calcium oxalate monohydrate or apatite, or (one calculus) it may approach a pure state (low per cent of apatite) (Figs. 666 and 667, *1, 4*).

In pure form it has a radially fibrous or bladed structure (Fig. 667, *2, 3*). A crudely divergent tufted or fernlike structure may sometimes be seen in cross section (Fig. 667, *2*). The ends of these tufts produce a uniformly nodular appearance (Fig. 667, *1*).

Its color is creamy white or yellowish white, ocasionally light brown; structure, moderately soft. Fracture surfaces tend to have a somewhat pearly or silky luster, due to perfect cleavage. It differs from fibrous calcium oxalate monohydrate in this characteristic and in that the latter is harder, darker, and lacks a good cleavage along the length of its fibers.

They are monoclinic in crystallization, with perfect cleavages on both (010) and (301).

Hardness 2-2½
Specific gravity 2.32
Optical properties:
  $X = 1.539$
  $Y = 1.545$
  $Z = 1.551 = b$
  Biaxial positive (+)
  $2V = 87°$
  Dispersion perceptible $v < r$
  Extinction $Y \wedge a = 18°$

Microscopically, its low birefringence is approximated by magnesium ammonium phosphate hexahydrate and calcium oxalate dihydrate from which it is easily distinguished by its higher indices and bladed crystals.

Uric acid ($C_5H_4N_4O_3$)

In calculi uric acid occurs most often in a pure state. Its most common associates in calculi of mixed composition are apatite and calcium oxalate monohydrate. It occurs sometimes as the nucleus in calculi composed of calcium oxalate monohydrate or of apatite. Apatite may also be found in the interstices of coarsely porous uric acid calculi. Calcium oxalate monohydrate has also been found as the nucleus of a uric acid stone.

They have a dense fine-grained structure and are relatively heavy (Fig. 668, *1*); oblate or flattened pebblelike shape with smooth but not polished surface is typical; warty surfaces

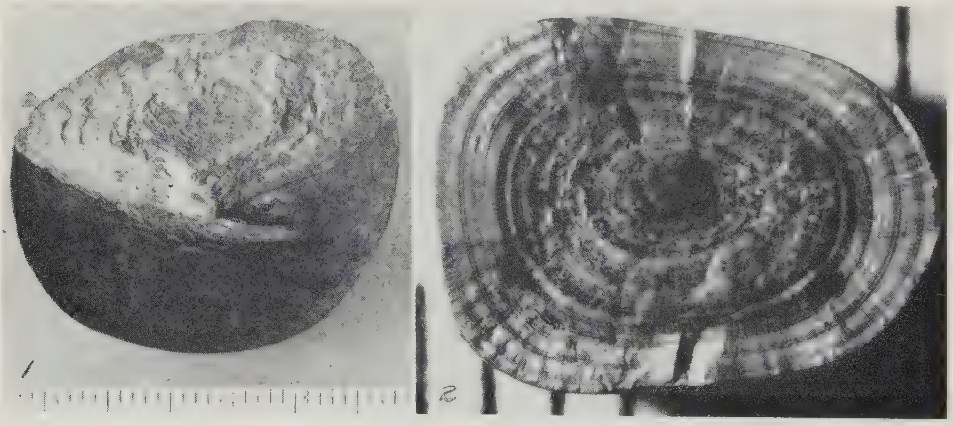

Fig. 668. Uric acid calculi. **1,** Dense fine-grained structure shown on fractured surface. **2,** Alternating light and dark laminations are all pure granular uric acid. Central nucleus of uric acid crystals. Scale in millimeters. (From Prien, E., and Frondel, C.: J. Urol. **57:**949, 1947.)

may be observed. Cross sections usually show a concentric lamination of variable coarseness with radial striation (Fig. 668, *2*). Alternating light and dark laminations are of identical composition, the dark layers probably being due to pigmentation. Some calculi have a granular appearance without evidence of lamination. Nucleus is usually composed of unoriented uric acid crystals. Its color is some shade of brown with which red or orange may be mixed.

It is orthorhombic in crystallization and has good cleavage. Hardness 2½; specific gravity 1.89. Some crystals are rectangular laths or rounded lozenge-shaped tablets flattened on (001) (Fig. 673, *9, 10*). Optically it is biaxial. Optical sign is unknown. Optic plant is parallel to (001), with $X = 1.573$, $Y = ?$, $Z = 1.830$. Polymorphous or hydrated form, stable at relatively low temperature, is said to have $X = 1.53$ and $Z = 1.73$. Pure recrystallized uric acid is white to yellowish white and pale brownish white. It has a marked tendency to adsorb foreign pigmenting substances; the pigmented material usually is pleochroic under the microscope, with absorption $Z > X$. The so-called "brick dust" precipitate from urine is pigmented uric acid.

Microscopically the high birefringence of uric acid is approached only by calcium oxalate monohydrate among the common stone-forming substances. It is distinguished from the latter by finding grains that have indices of refraction above 1.65 (the highest index of calcium oxalate monohydrate) and failure to find grains with indices below 1.573 (the lowest index of uric acid).

Urates: Many urates, including both the acid and normal salts of NH_4, Na, K, and Mg, have been described in urinary calculi. Identification is questionable or inadequate. Urates occur less commonly in calculi than would appear from the results of chemical analysis alone. Sodium acid urate has been found but once and in microscopic amount in a series of approximately 700 urinary calculi. It has been found by Prien in several tiny plaques attached to the apices of renal papillae and in the so-called uric acid infarcts of renal papillae. In the single occurrence in a calculus a large central nucleus of uric acid was surrounded by a dark gray-brown zone with a radially fibrous structure; a peripheral layer of apatite was present (Fig. 669). Dissection showed the soft fine silky needles of sodium acid urate easily visible under magnification (Fig. 670).

Sodium acid urate ($NaC_5H_3O_3N_4 \cdot H_2O$) is

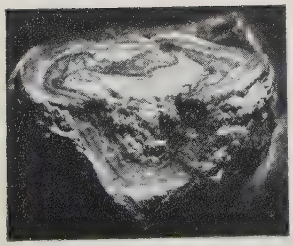

Fig. 669

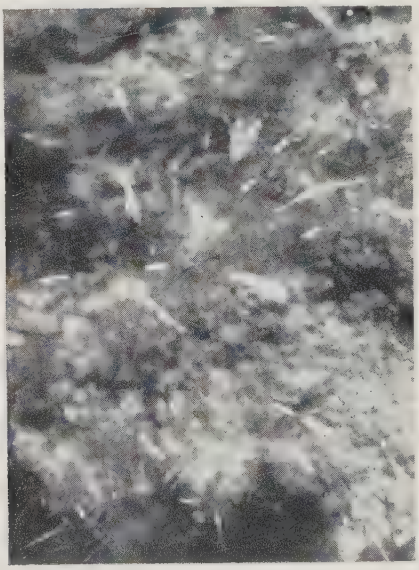

Fig. 670

Fig. 669. Sodium acid urate in fractured uric acid calculus. Narrow dark zones extending around calculus composed of needlelike crystals of sodium acid urate which extend across zone. Diameter of calculus 0.4 cm. (From Prien, E., and Frondel, C.: J. Urol. 57:949, 1947.)

Fig. 670. High-power view of sodium acid urate crystals seen upon fractured surface of calculus in Fig. 669. (×30.) (From Prien, E., and Frondel, C.: J. Urol. 57:949, 1947.)

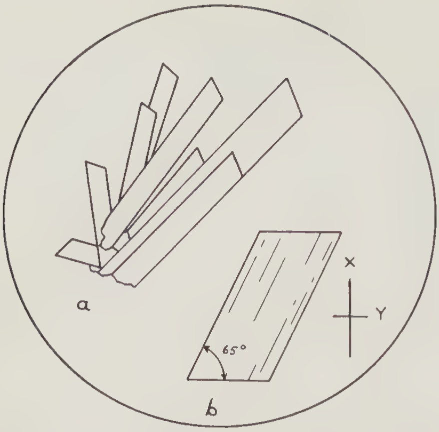

Fig. 671. Sodium acid urate crystals. (×315.) (From Prien, E., and Frondel, C.: J. Urol. **57**:949, 1947.)

monoclinic or triclinic in crystallization. Crystals are lath or needle-shaped and possess 2 perfect cleavages and probably a third imperfect cleavage. Material is very soft and plastic.

Its color is white, yellowish white, or cream yellow. Crystal aggregates have a silky luster. Microscopically crushed fragments appear as rhomboidal plates or laths bounded by cleavage faces with extinction parallel to one of the cleavages. Optically positive (+) with X = 1.448, Y $\sim$ 1.75, Z $>$ 1.84. Interior acute angle of the rhomboidal plates is approximately 65°, as shown in Fig 671.

Ammonium acid urate was found in a bladder stone from a bulldog.

Another substance was found once in microscopic amount in a depression upon the surface of a calcium monohydrate calculus. It remains unidentified but was probably a urate.

Cystine ($SCH_2CH(NH_2)$—COOH)

Cystine usually occurs in the pure state in calculi, but sometimes it is found admixed with small amounts of apatite and, rarely, calcium oxalate monohydrate. Occasionally it may exist in a pure state as the nucleus of a calculus otherwise composed of the above constituents.

Cystine stones commonly consist of porous aggregates of relatively well-formed short hexagonal prisms or hexagonal tablets (Fig. 672). Well-crystallized stones sometimes have a compact granular center. The color is honey-yellow to yellowish white. The luster is waxy. The calculi are very soft unless they are densely granular. An occasional cystine calculus may be so fine-grained and dense as to resemble uric acid externally.

Cystine is hexagonal in crystallization and possesses a perfect cleavage on (0001).

Hardness 2
Specific gravity 2.06
Optical properties:
  O = 1.700
  E = 1.640
  Uniaxial negative (−)

Cystine crystals commonly have the form of hexagonal tablets, as shown in Fig. 673, *5*. The substance is easily recognized under the microscope, affording grains with a hexagonal outline which yield a centered uniaxial negative interference figure, with O = 1.700. The crystals and cleavage fragments usually show internal growth bands parallel to the external hexagonal crystal faces.

Other substances

Prien failed to find xanthine and indigo in his study. It seems reasonable to predict that small amounts of other substances may be detected in calculi by physical methods. Cholesterol has been reported. Prien has found cholesterol crystals in urine and in gallstones but not in calculi.

Calcium carbonate ($CaCO_3$—aragonite; calcite; vaterite-B or μ-calcite)

Calcium carbonate is commonly reported to occur in phosphatic calculi. Prien et al. have not encountered it. Development of effervescence when a calcareous stone is treated with dilute acid is not necessary evidence for the occurrence of calcium carbonate.

Calcium carbonate crystallizes in 3 distinct forms and may be said to be polymorphous. The phenomenon is due to the geometrically different manner in which the constituent atoms are arranged in the crystal structure of the 3 substances. As a consequence the different forms may vary markedly in physical

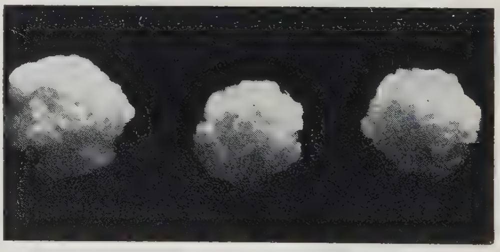

Fig. 672. Cystine calculi composed of aggregates of hexagonal crystals. Soft pearly luster difficult to photograph. Diameter of calculi 2-3 mm. (From Prien, E., and Frondel, C.: J. Urol, **57**:949, 1947.)

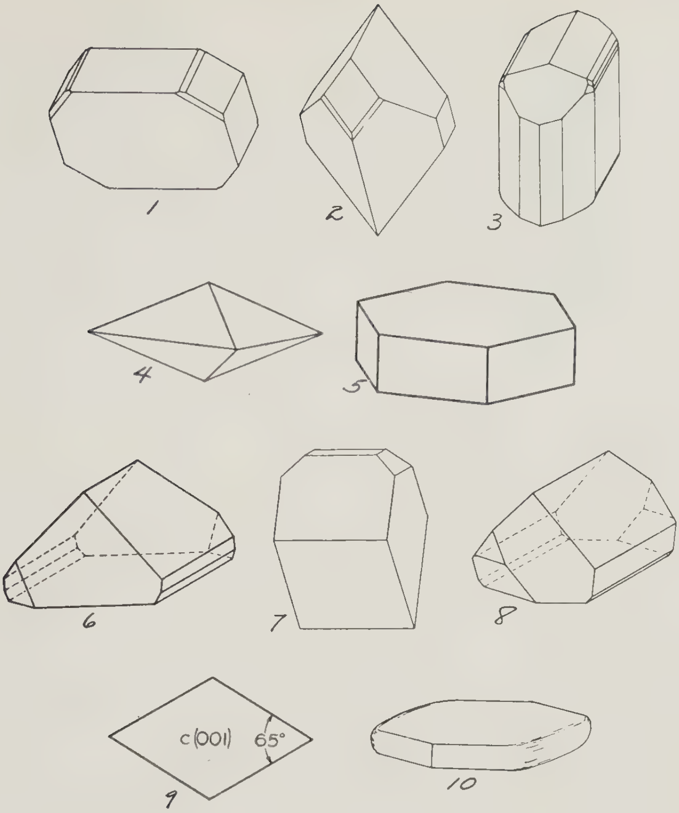

Fig. 673. Forms of crystals seen upon the surfaces of calculi. **1-3**, Calcium oxalate monohydrate, uncommon, actual size 0.03-0.1 mm. **4**, Calcium oxalate dihydrate, common, actual size up to 2 mm. **5**, Cystine, common, actual size 0.1-0.2 mm. **6-8**, Magnesium ammonium phosphate hexahydrate, common, actual size 0.5-1 mm. **9**, **10**, Uric acid, uncommon, actual size 0.2 mm. (From Prien, E., and Frondel, C.: J. Urol. 57:949, 1947.)

and chemical characters. A common example is provided by diamond and graphite, both of which are pure carbon. Calcium carbonate occurs as aragonite, calcite, and vaterite-B or μ-calcite. All 3 phases have been recognized as constituents of gallstones. So far as is known no specific phase has been identified in human urinary calculi. Calcium carbonate concretions are said to occur in herbivora.

Distinction between the 3 calcium carbonate phases may be effected optically if the grain size is sufficiently large or, in any case, by x-ray powder patterns. Aragonite, and possibly calcite as well, would probably appear as fine-grained or fibrous aggregates.

Aragonite: Aragonite is orthorhombic in crystallization with a distinct cleavage on (010) and indistinct cleavages on (110) and (011).

Hardness 3½-4
Specific gravity 2.95
Optical properties:
$X = 1.530 = c$
$Y = 1.680 = a$
$Z = 1.685 = b$
Biaxial negative (−)
$2V = 18°$
Dispersion $r > v$, weak

Calcite: Calcite is rhombohedral in crystallization and has a good rhombohedral cleavage on (1011).

Hardness 3
Specific gravity 2.715
Optical properties:
$O = 1.658$
$E = 1.486$
Uniaxial negative (−)

The E′ index afforded by grains resting on cleavage surfaces is 1.566.

Vaterite-B or μ-calcite: Vaterite-B is hexagonal in crystallization and occurs in artificial preparations as tiny hexagonal or spherulitic aggregates.

Optical properties:
$O = 1.550$
$E = 1.640$
Uniaxial positive (+)

Table 364. *Determinative table of optical constants of urinary calculi*

Name	*Crystal system*	*Sign*	*X or O*	*Y*	*Z or E*	*2V*	*Dispersion*	*Extinction angle*	*Distinguishing features*
Calcium oxalate monohydrate (whewellite)	Monoclinic	+	1.491 = *b*	1.555	1.650	84°	r < v weak	Z ∧ *c* = 31°	Distinguished from uric acid by indices; no index above 1.650; has index below 1.573
Calcium oxalate dihydrate (weddellite)	Tetragonal	+	1.523		1.544				
Magnesium NH_4PO_4 hexahydrate (struvite)	Orthorhombic	+	1.495 = *a*	1.496 = *c*	1.504 = *b*	37°		r < v strong	
Carbonate-apatite	Hexagonal	Isotropic	Index variable, usually 1.55-1.59 (extreme range 1.520-1.610)						Isotropic; spherulitic; effervesces with HCl
Hydroxyl-apatite	Hexagonal	Isotropic	Index variable, as above						Isotropic; spherulitic; no effervescence with HCl
Calcium hydrogen PO_4 dihydrate (brushite)	Monoclinic	+	1.539	1.545	1.551 = *b*	87°	v < r perceptible	Y ∧ *a* = 18°	Coarse radial fibers
Uric acid	Orthorhombic	?	1.573	?	1.830			Parallel to cleavage	Yellow color; see under calcium oxalate monohydrate
Sodium acid urate	Monoclinic or triclinic		1.448	1.75	1.84			Parallel to cleavage	Rare; needlelike crystals with internal acute angle = 65°
Cystine	Hexagonal	–	1.700		1.640				Hexagonal crystals; good cleavage
Tricalcium phosphate (whitlockite)	Hexagonal	–	1.629		1.626				Rare; like apatite

Calcium sulfate dihydrate (gypsum—$CaSO_4 \cdot 2H_2O$)

Calcium sulfate has been said to occur occasionally as a urinary deposit but has not been observed in calculi by Prien et al. Gypsum, the particular phase which would be stable as a urinary deposit, is monoclinic in crystallization. Hardness 2; specific gravity 2.32. Gypsum has a perfect cleavage on (010) and imperfect cleavages on (111) and (100). Optically gypsum is biaxial positive, with $X = 1.521$, $Y = 1.523$, $Z = 1.530$, and $2Z = 58°$. The optic plane is parallel (010), the direction of perfect cleavage. Strongly inclined dispersion, with $r > v$.

Calcium acid phosphate (monetite—$CaHPO_4$)

Monetite has been reported as a urinary sediment in carnivorous animals but has not been reported from human beings. Crystals are rhomboidal plates of laths and are triclinic in crystallization. Optically monetite is biaxial positive (+), with moderately large 2V, and $X = 1.600$, $Y = 1.614$, $Z = 1.631$. In comparison to brushite, $CaHPO_4 \cdot 2H_2O$, monetite forms artificially at relatively higher temperatures from acid solutions.

Cholesterol ($C_{27}H_{46}O$)

Cholesterol has been described in urinary calculi; it occurs rarely as a heavy suspension of crystals in urine and in hydrocele fluid. It crystallizes in thin, transparent rhomboidal plates of varying size, often imbricated and united to one another. A rhomboidal notch frequently exists at one corner. The crystals should be recognizable at sight. Melting point 147.5°-148.5°. Perfect cleavage (010). Optical properties: monoclinic in crystallization. $Z = 1.566$, $Y = 1.532$, $X = 1.520$, $2V = 60° \pm$.

Tribasic magnesium phosphate hydrate (bobierrite—$Mg_3(PO_4)_2 \cdot 8H_2O$)

Magnesium phosphate has been said to occur rarely as a urinary deposit but has not been observed by Prien et al. The phase stable as a urinary deposit probably is the octahydrate, bobierrite. This substance is monoclinic in crystallization, with a hardness of 2 and a specific gravity of 2.2. Optically biaxial positive, with $X = 1.510$, $Y = 1.520$, $Z = 1.543$. $2V = 71°$, weak dispersion, with $r < v$. Extinction angle Z to c = +29°. Optic plane perpendicular to (010).

CHEMICAL ANALYSIS OF URINARY CALCULI

In examining the larger urinary calculi chemically, the most valuable data are obtained by examining each of the concentric layers separately. Saw the calculus through the nucleus and separate the various layers. Material may also be obtained by scraping enough powder from each layer to carry out the examination. If the latter is adopted, the layers should not be separated.

Murexide test

To a small amount of unknown material in a small evaporating dish, add 2 to 3 drops of concentrated nitric acid. Evaporate to dryness over a water bath. If uric acid is present, a red or yellow residue remains which turns purplish red after cooling the dish and adding a drop of very dilute ammonium hydroxide. The color is due to the formation of ammonium purpurate or murexide. If potassium hydroxide is used instead of ammonium, a purplish violet color due to the production of the potassium salt is obtained. The color disappears upon warming; with certain related bodies (purine bases) the color persists under these conditions.

Table 365 shows a scheme proposed by Heller for the chemical examination of urinary calculi and will be found very useful in determining their composition. Reduce the calculus to powder and proceed as directed.

Kamlet method

Table 365 is designed to give an outline of the pure chemical analysis of calculi. A very useful technic has been introduced by Kamlet.[33] In this method, as in the one just described, the larger calculi are cut up, and each of the concentric layers is subjected to a separate analysis. Small calculi are examined in toto. If possible, 100 mg. of powdered material are examined.

1. Describe the calculus as to shape, size, color, weight, and appearance on transection.
2. Divide the specimen into 2 equal parts for analysis. Reserve one portion for the detection of cystine, xanthine, urostealith, fibrin, cholesterol, and indigo. Macerate the other portion to a fine powder in a clean, dry porcelain mortar and add to 50 ml. 10% hydrochloric acid (sp. gr. 1.05) in a small Pyrex flask. Effervescence at this point indicates the presence of carbonate ion. Gently boil the mixture for 5 min., filter through ashless filter paper, and distribute the filtrate evenly into 5 clean test tubes 6 × 1 in.
3. Render 1 tube faintly alkaline with 10% sodium hydroxide solution and add 5 ml. Nessler reagent. A brown turbidity or sediment (the iodide of Millon base) indicates the presence of ammonium ion.
4. Render 1 tube faintly alkaline with 10% sodium hydroxide solution and then cautiously reacidify with dilute acetic acid. Add 1 ml. arsenophosphotungstic acid and 4 ml. 5% sodium cyanide solution. A blue color develop-

Table 365. *Chemical examination of urinary calculi**

On heating powder on platinum foil, it									
Does not burn				Does burn					
The powder when treated with HCl				With flame			Without flame		
Does not effervesce								Powder gives murexide test	
Powder gently heated, then treated with HCl								Powder when treated with KOH gives	
Powder when moistened with a little KOH									
Abundant ammonia; powder dissolves in acetic acid or HCl; solution gives crystalline precipitate with ammonia	No ammonia or at least only traces of ammonia; powder dissolves in acetic acid or HCl; solution is precipitated by ammonia (amorphous)	Effervesces	Effervesces	Flame yellow, continuous; odor of burnt feathers; insoluble in alcohol and ether; soluble in potassium hydroxide with heat; precipitated herefrom by acetic acid and generation of hydrogen sulfide	Flame yellow, pale, continuous; odor of resin or shellac on burning; powder soluble in alcohol and ether	Flame pale blue, burns a short time; peculiar sharp odor; powder dissolves in ammonia; 6-sided plates separate on spontaneous evaporation of ammonia	Does not give murexide test; powder dissolves in nitric acid without effervescence; dried yellow residue becomes orange with alkali, beautiful red on warming	Strong ammonia reaction	No noticeable ammonia reaction
"Triple phosphate" (mixed with unknown amount of earthy phosphate)	Bone-earth (magnesium and calcium phosphate)	Calcium oxalate	Calcium carbonate	Fibrin	Urostealith	Cystine	Xanthine	Ammonium urate	Uric acid

*Scheme proposed by Heller; from Hawk and Bergeim, ed. 6, Philadelphia, P. Blakiston's Son & Co.

ing within 60 sec. indicates uric acid or urate ion.

5. Render 1 tube faintly alkaline with 10% sodium hydroxide solution and then reacidify with dilute acetic acid. A white turbidity or precipitate indicates the presence of calcium oxalate. If no turbidity results after standing for 10 min., add 5 ml. saturated ammonium oxalate solution. A white precipitate or turbidity at this point indicates the original presence of calcium ion only (probably as calcium carbonate).
6. Render 1 tube ammoniacal with strong ammonia water and add 1 ml. 0.5% solution of of *p*-nitrobenzeneazoresorcinol in 95% alcohol. A blue color developing within 30 sec. indicates the presence of magnesium ion.
7. Add to the last tube 2 ml. acid molybdate reagent (equal parts of 10N sulfuric acid and 7.5% sodium molybdate solution) and 1 ml. dilute tin chloride ($SnCl_2$) solution (1 ml. of a solution of 10 gm. $SnCl_2$ in 25 ml. concentrated hydrochloric acid diluted with 200 ml. water). A blue color developing within 60 sec. indicates the presence of phosphate ion.
8. Agitate the residual half of the specimen for analysis with 10 ml. chloroform. An indigo calculus will turn the chloroform blue. The chloroform is then decanted from the insoluble sediment.
9. Evaporate a few drops of the chloroform extract on a glass slide and stain the residuum with Sudan III. Residual fat globules on the slide indicate a urostealith calculus.
10. Add to the remainder of the chloroform solution 5 ml. acetic anhydride and 0.5 ml. concentrated sulfuric acid. A Liebermann-Burchard reaction (play of colors ending in a naphthol green shade) indicates the presence of cholesterol.
11. Divide the insoluble sediment from step 8 into 3 parts. Dissolve 1 part in nitric acid and cautiously dry over a free flame. A yellow residue which turns orange on the addition of 10% sodium hydroxide solution and deep red on warming indicates the presence of xanthine.
12. To the second part of the insoluble sediment from step 8 add 2 ml. Millon reagent (p. 1809) and heat. A positive protein reaction (red precipitate) indicates the presence of fibrin in the calculus.
13. To the last part of the insoluble sediment from step 8 add 2 ml. concentrated ammonia water and agitate for 2 or 3 min. Allow 1 drop of the resultant solution to evaporate on a slide.

If cystine is present, 6-sided platelets will separate on the spontaneous evaporation of the ammonia. For a confirmatory test, dissolve 5-10 mg. of powdered calculus in 5 ml. N/10 hydrochloric acid, add 2 ml. 5% sodium cyanide solution, mix, and let stand 10 min. Add 1 ml. 0.5% aqueous solution of 1:2-naphthoquinone-4-sodium sulfonate and 5 ml. 10% solution of sodium sulfite (Na_2SO_3) in N/2 sodium hydroxide solution. Mix and let stand for 30 min. In the presence of cystine a reddish brown color forms and will turn deep red on the addition of 1 ml. 2% solution of sodium thiosulfate ($Na_2S_2O_4$) in N/2 sodium hydroxide solution.

Chapter 103

Kidney function

SAM FRANKEL

There are a number of tests for the estimation of kidney function. Before proceeding to outline the manner of performance of these tests, some fundamental facts regarding the physiology of the kidney must be reviewed. For those who desire an exhaustive and timely knowledge of the true physiology of the kidney, it is suggested that the textbook of Homer W. Smith, *The physiology of the kidney,*[34] be carefully read. Attention is to be called to the diagram from Smith's book (Fig. 674) showing the essential features of the typical nephron in the human kidney. This anatomic study of the nephron or unit of kidney structure shows the glomerulus in which the formation of urine begins. The glomerulus is a tuft of capillaries supplied with blood through an afferent arteriole. The capillary tuft is thrust into the expanded but closed end of the tubule so far that the tuft has come to be enveloped by a double layer of tubular epithelium, the inner or visceral layer being closely applied to the capillaries, extending in between all the loops and surrounding each loop almost completely, and the outer or parietal layer forming a smooth spherical capsule, Bowman's capsule, about the tuft of capillaries. Any fluid passing through the capillaries enters the capsular space around the tubule. This is lined with a thin glomerular basement membrane, anatomically continuous with the basement membrane bounding the outer surface of the tubule cells. The tubule shows a convolution near the glomerulus, known as the proximal convoluted tubule, passes by a more or less straight course toward the pelvis of the kidney, and abruptly reverses its direction, forming what is called Henle's loop. It then returns to the region of its own glomerulus where it undergoes the second series of convolutions, the distal convoluted tubule, and later joins a collecting duct. The proximal and distal convoluted segments of one tubule are closely intertwined in one locality.

It is believed that the process that takes place in the glomerulus is pure filtration. The capsular fluid contains all the filtrable constituents of blood plasma in the same concentration per unit volume of water as these are present in the plasma, according to Smith, "except for such inequalities in the distribution of ions as might arise from the presence of nonfiltrable proteins."

We know that the substances that are present in the plasma but absent from the urine must be reabsorbed by the tubules as the glomerular filtrate passes along them on its way to the bladder. A large portion of the water present in the filtrate is also reabsorbed by the tubules. This is evidenced by the fact that products such as creatinine and urea may be present in the urine in many times the concentration that they are present in the plasma. According to Smith, in every man about 90 ml. filtrate are formed in the kidneys every minute, of which 89 ml. water are reabsorbed by the tubules, leaving 1 ml. of urine to be excreted, even under conditions of relatively high urine flow. Variations in urine flow are referable primarily to variations in the amount of water reabsorbed rather than to the variations in the amount of filtrate formed.

Some consider that the glomerulus is a filter for urea nitrogen and therefore tests for identifying urea nitrogen in blood and urine are tests of glomerular function. The proximal convoluted tubule must be considered as that part of the kidney structure that is concerned with the reabsorption and elimination of other

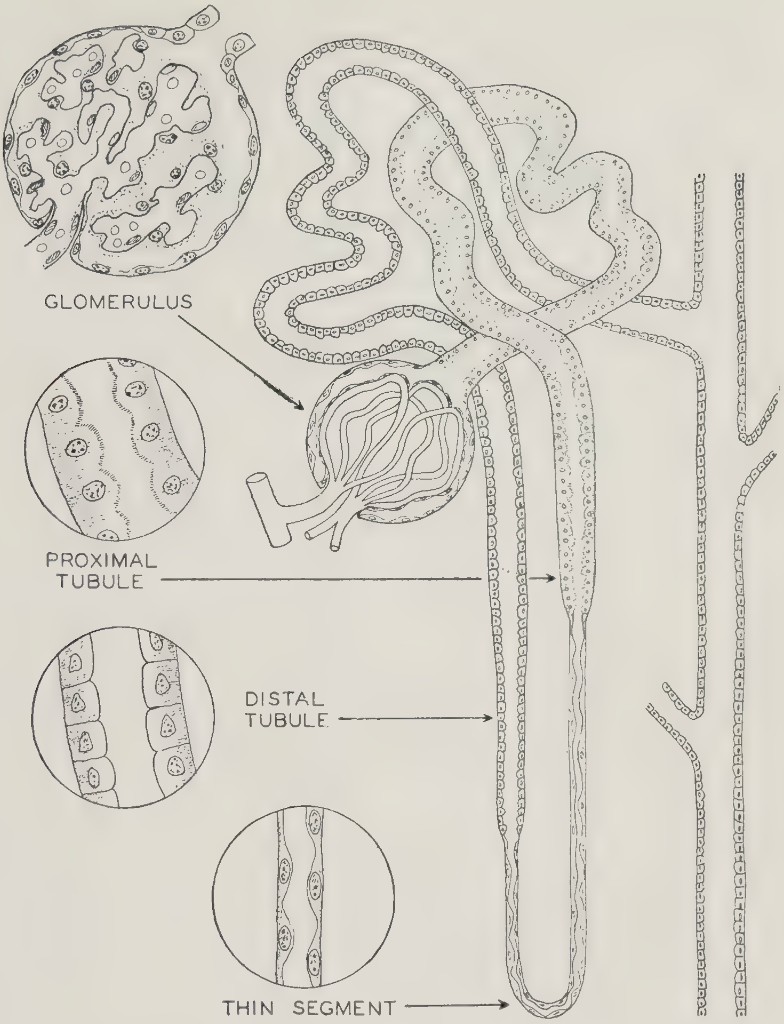

Fig. 674. Diagram showing essential features of a typical nephron in human kidney. (From Smith, H. W.: The physiology of the kidney, London, 1937, Oxford University Press.)

components of urine. Tests for concentration of urine are therefore used to demonstrate the function of the proximal convoluted tubules of the kidney.

There are a number of physiologic factors in connection with these 2 elementary principles. First, the glomerular blood pressure is of importance in relation to effective filtrate pressure. From a number of experimental observations it has been shown that increase of pressure in the renal circulation causes corresponding increase in urine formation. This supports the evidence derived from anatomy that the glomerulus is the structure in which changes in pressure operate to cause changes in urine volume. Furthermore, the contractility of the efferent vessel is of importance in regard to the regulation of glomerular filtration. Another factor involved in the maintenance and adjustment of glomerular capillary pressure and associated with the filtration of cell-free, protein-free fluid from the blood as it flows through the glomerular capillaries is viscosity. The number of cells per unit volume in the blood in the efferent arteriole is higher than that in the afferent. This is a factor that must vary with the rate of blood flow, volume of glomerular filtrate, and caliber of vessels.

The function of the proximal tubule is normally bound up with the process of reabsorption. Wearn and Richards[35] first showed by puncture of the capsule of Bowman that the urine from that portion contained sugar, whereas urine issuing from the ureter contained none: hence the principle of reabsorption was established. At the same time Bieter and Hirschfelder[36] showed that when they injected phenol red into frogs, the contents of the tubules were more intensively stained than were the contents of the capsule of Bowman. This observation was presented by them as reabsorption of water from the tubules. From experiments with phenol red it was shown that it is a substance the excretion of which cannot be accounted for by glomerular filtration. In other words, this substance which cannot be diffused through the walls of the tubule must be secreted in the proximal portion.

Expressed in other terms, the glomerular portion of the kidney is able to eliminate practically all soluble and diffusible substances which may enter the blood stream. This process is in line with the filtration theory of glomerular function. The evidence is definite that reabsorption takes place in the proximal portion of the tubule. Urinary secretion consists of glomerular filtration, tubular reabsorption, and tubular secretion, with possible evidence of some amount of glomerular secretion. It is thought that the formation of the urine is effected through the following processes:

1. Ultrafiltration of large amounts (for the human kidney, about 150 ml./min.) of protein-free fluid in the glomeruli.
2. Active reabsorption of water.
3. Active reabsorption of threshold bodies, like sugar and chlorides.
4. Passive back diffusion of all other substances in degrees varying according to the permeabilities of the tubule walls for each—with creatinine at one end as the most highly concentrated and alcohol at the other end as a substance not concentrated at all.
5. Secretion of substances not preformed in the blood.

Regarding the **excretion of sugar in the urine,** it has been known for a long time that whenever the quantity of glucose in the blood increases, sugar is likely to be found in the urine. This occurs when there is failure on the part of the blood to metabolize carbohydrates. Sugar filters out through the glomerulus, and its presence in the urine must be due to the fact that the tubules fail to reabsorb it rather than to any impairment of renal function. The threshold point for sugar is generally supposed to be around 170 mg.%, although in some cases it is as high as 210 mg.%. In other words, the threshold point in man is variable. In renal diabetes the threshold point is lowered, and sugar appears in the urine with a normal plasma glucose level.

As Smith states, "The nature of the glucose 'threshold' mechanism of the kidney can be examined by the simultaneous determination of the rate of glucose filtration and the glucose clearance. The quantity of glucose filtered per minute will be the filtration clearance (in the dog, the creatinine clearance) multiplied by the concentration of glucose in the plasma; the difference between this quantity and the quantity excreted per minute gives the quantity reabsorbed by the tubules. The glucose clearance is essentially zero until the plasma level reaches a critical value (the 'threshold'); above this level glucose begins to be excreted, the clearance increasing rapidly with rising plasma level and approaching the filtration rate as its asymptote. Ni and Rehberg,[37] assuming that reabsorption takes place in the proximal tubule before any reabsorption of water occurs, calculated the equilibrium concentrations in the blood and urine at various levels of hyperglycemia and concluded that the reabsorptive process is limited by a maximal concentration difference between these two fluids. They recognized, however, that the quantity of glucose which the tubule cells could reabsorb per minute might have some upper limit. But in point of fact, so long as the rate of filtration remains constant, the reabsorption of a constant quantity of glucose will lead to a constant concentration difference between tubular urine and blood, under their assumption, so that this latter relationship may be purely fortuitous."*

Glucose is almost completely reabsorbed by the tubules when it is present in the glomerular filtrate in the same concentration as in blood plasma. If, however, the plasma level is elevated, part of the glucose is not reabsorbed and escapes into the urine.

The **transit of urea from the blood into the urine** seems to occur in the glomerulus. There is no evidence at this time that there is any active reabsorption of urea in the sense that glucose is reabsorbed by the tubules. A test for glomerular function consequently must be the urea clearance test, which is fully explained on p. 74. **Urea clearance** means the volume of blood cleared of urea by 1 min. excretion of urine. An adult with a normal volume of urine excretes per minute the amount of urea in 75 ml. blood. According to Smith, "the deficit in the urea clearance is due to the escape of urea from the lumen of the tubules back into the blood in consequence of a high concentration gradient established by the reabsorption of water, supplemented by an as yet unknown reabsorptive process which takes up something like 40% of the filtered urea at the highest urine flow obtainable. During the time when the urine flow is increasing, the urea clearance may be raised to abnormally high values, relative to the rate of urine formation. In view of the multiplicity of the factors influencing the rate of excretion of urea, it appears best to adhere to the empirical description of this process in terms of standard and maximum clearances, using the latter wherever practicable."*

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*From Smith, H. W.: The physiology of the kidney, London, 1937, Oxford University Press.

Another method is the **Ambard coefficient.** In this test the rate of urea excretion in the urine varies directly as the square of the urea concentration in the blood. With urea concentration of the blood remaining constant while that of the urine varies, the rate of urea excretion of the urine is inversely proportionate to the square root of its concentration in the urine. Furthermore, if both the blood urea concentration and the concentration of urea in the urine are variable, the rate of urea excretion varies directly as the square of urea concentration in the blood and inversely as the square of urea concentration in the urine. Ambard[38] has given us his formulas as follows:

$$\frac{\text{Urea in blood}}{\text{Rate of excretion}} = \text{Constant, or}$$

$$\frac{\sqrt{\text{Urea in blood}}}{\sqrt{\text{Excretion/unit of time}}} = \text{Constant}$$

Again, the

$$\frac{\text{Rate of excretion I}}{\text{Rate of excretion II}} = \frac{\sqrt{\text{Concentration II}}}{\sqrt{\text{Concentration I}}}$$

Expressed by Mosenthal and Lewis,[39] the formula is as follows:

$$K = \frac{Ur}{\sqrt{D \times \frac{70}{P} \times \frac{C}{25}}}$$

In which K = the coefficient of urea excretion.
Ur = urea gm./L. blood.
D = urea gm. excreted in urine in 24 hr.
C = urea gm./L. urine.
P = body weight in kg.
70 = standard body weight in kg.
25 = standard concentration of urea gm./L. urine.

K, or the coefficient of urea excretion, lies between 0.06 and 0.09 for a body weight of 70 kg., with an average of 0.08.

The constant is unaffected by diet. With increasing degrees of renal impairment the constant rises. A constant above 0.09 indicates impairment of urea excretion; in fact, in severe grades of nephritis, values of 0.30 to even 1.0 may be encountered. Quoting from Mosenthal and Lewis,[39] "When the values rise above 0.09, some impairment of the power of the kidney to excrete urea is indicated. Inability of the kidney to eliminate urea in proportion to the concentration of the blood urea results in an increase in Ambard's coefficient. In a normal individual it will remain within the limits mentioned, no matter what the height of blood urea; in persons with impaired renal function, however, the kidney does not answer the diuretic stimulus of the blood urea adequately, too little urea is put out, and the result is a rising coefficient, whether the urea in the blood be high or low. This test of Ambard is important in the detection of early kidney functional deficiency."*

Van Slyke and Cullen[40] conclude as follows regarding the Ambard coefficient:

> "1. The Ambard formula (original or modified by McLean) is not a precise expression of the law of renal function insofar as elimination of urea is concerned. This is particularly true as regards the effect of urinary urea concentration.
>
> "2. The upper limit of blood urea in normal individuals taken under normal circumstances is 0.35 gm. urea per liter of blood. Higher figures, under normal conditions, indicate evidence of impaired renal function.
>
> "3. With McLean's modification of Ambard's formula, in the majority of nephritic cases, a lowering of the index is accompanied by a rise of blood urea above the 0.35 gm. per liter limit. The index affords no valuable diagnostic or prognostic information which one cannot deduce from the blood urea alone.
>
> "4. In some instances, for example, arteriosclerosis and cardiac decompensation, blood urea was normal with a lowered index. Since it is important to distinguish between vascular and renal cases, the index loses its clinical value.
>
> "5. There is possibility of error from undetected incomplete collection of the urine. This cannot occur in a simple blood urea estimation.
>
> "6. Urea index estimations in the same individual yield wider variations in normal or nonnephritic individuals than in nephritics.
>
> "7. The estimation of blood urea is more useful for ordinary clinical diagnosis and prognosis than the estimation of the urea index or the Ambard quotient."†

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*From Smith, H. W.: The physiology of the kidney, London, 1937, Oxford University Press.

*From Mosenthal and Lewis: J.A.M.A. **67**:933, 1916.

†From Van Slyke and Cullen: J. Biol. Chem. **19**: 211, 1914.

Usefulness of blood chemical tests: It is true that there may be severe impairment of kidney function with a normal blood chemistry because the patient has so adjusted his food intake as not to exceed the functional capacity of the kidney or because the diminished concentrating capacity of the kidney has been balanced by a relative polyuria. For the estimation of the impairment of renal function, particularly so far as the glomerular function is concerned, blood chemical methods alone constitute the laboratory criterion of accumulation of the urinary constituents in the blood. The substance in which we are the most interested is the accumulation of urea. The accumulation of total nonprotein nitrogen is not nearly so important in this regard as is the accumulation of urea because there is much more nonprotein nitrogen in the tissues than in the blood. The estimation of the other nonprotein nitrogenous substances, such as uric acid, creatinine, indican, etc., although important, may be routinely dispensed with in the estimation of kidney function. The interpretation of the urea content of the blood must be guided somewhat by the previous diet of the patient. This was well illustrated by von Dolivo,[41] who found that in the normal person 2 hr. after the ingestion of 20 gm. urea the urea content of the blood rises to about 40 mg.% above the initial value. It then declines, to reach the normal in somewhat less than 24 hr. He found that patients with nitrogen retention reach a maximum more slowly, attaining it in about 4-6 hr., the increase in urea concentration being 60-70 mg.%. In addition to this, patients with renal insufficiency require 2-3 days to return to the original values. King[42] obtained similar results, according to studies he made on the effect of the ingestion of urea on the level of this substance in the blood.

It is evident, therefore, that while making chemical tests of blood, data on the previous diet of the patient must be known. As Fishberg[43] states: "This is also illustrated in patients with the oliguria of chronic nephrosis who may have considerable elevation of the nonprotein nitrogen of the blood as a result of high protein diet and the administration of urea, even though renal function is unimpaired. The prognostic significance of nitrogen retention in one who has previously been on rigorous protein restriction is more serious than in one who has been eating meat and other nitrogenous foods at will."*

We know that urea nitrogen accumulates in the blood in renal insufficiency. The normal value is about 8-20 mg./100 ml. blood. This may rise to as much as 300 mg.%. Uric acid usually parallels urea, but exceptionally it may rise before urea begins to accumulate. Fishberg found hypertensive patients with elevated blood uric acid for protracted periods which did not indicate the onset of renal insufficiency. In general it may be stated that uric acid accumulation, indicative of kidney functional impairment, goes hand in hand with blood urea accumulation.

Creatinine is the last substance to accumulate in the blood following derangement of kidney function. It is therefore to be found in large quantities in the terminal stage of kidney function impairment.

Amino acids: There is generally no elevation of amino acids under circumstances of kidney failure. Elevated amino acid content of the blood has been found in the terminal stages of uremia, although there is no increase in ammonia as a result of impaired kidney function. Supposedly, the ammonia found in the kidney has its origin in the kidney, which explains the depletion of the alkali reserve in the body under certain conditions. If the efficiency of the kidney becomes impaired, the formation of ammonia is diminished, or if it does not keep pace with the rate of an excessive acid production, other fixed bases in the body must be called upon in the regulation of the body neutrality. In other words, in nephritis we have here an explanation of the increase in the intensity of the urinary acidity. It is to the study of urinary acidity that we must turn in our estimation of renal efficiency rather than to the study of data determined from an analysis of ammonia findings in the blood under these circumstances. The ammonia of urine, in other words, may be an index of impaired renal efficiency, but it is no index as to the degree of acid production. It is generally believed that the kidney is the seat of urinary ammonia formation.

Chlorides: There is usually a diminution in the amount of chlorides in blood in cases of impaired kidney function. This is due to loss of chlorides by vomiting and to ingestion of little or no salt. De Wesselow[44] found in advanced nephritis an inability of the kidney to excrete phosphoric acid, with resulting accumulation of the acid radical in the blood. Alterations in the inorganic blood chemical picture of the nephritic in the terminal stages of the disease are (1) a great increase in the phosphoric and sulfuric acid radicals and (2) since acids circulating in the blood are of necessity neutralized, abstraction of base from the plasma bicarbonate for combination with the acid excess occurs and the resulting volatile carbonic acid is excreted by the lungs. Though the diminution in the bicarbonate of the plasma is reasonably ascribed to the accumulation of phosphoric acid in the blood, it has been pointed out that the phosphate increase and the bicarbonate decrease are not in all cases proportional. It is probable, according to de Wesselow, that some form of redistribution of base must occur in which radicals other than phosphate play a part. In an observation on one case, de Wesselow noted that whereas the phosphate and the various basic radicals remain comparatively constant, the bicarbonate and chloride varied inversely. The rise in the bicarbonate content is accompanied by a fall in the chlorides.

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*From Fishberg, A. M.: Hypertention and nephritis, Philadelphia, 1934, Lea & Febiger.

In the vomiting of intestinal obstruction an extreme fall in the chloride content of the blood occurs and at the same time the plasma bicarbonate rises to a figure above the upper limit of the normal. These changes occur probably in the following way: Large quantities of hydrochloric acid are lost in the vomitus; this hydrochloric acid is derived in the process of gastric secretion from the plasma chloride, the residual sodium ions being balanced by bicarbonate. Under normal conditions the bicarbonate thus formed passes into the intestines with the pancreatic secretions and combines with the gastric hydrochloric acid to form sodium chloride, which can be reabsorbed. With vomiting the circle is broken, and, although the chloride content of the blood falls, the bicarbonate content rises.

It would appear then that the vomiting of uremia may be regarded as constituting a protective mechanism. By removal of hydrochloric acid, base is provided and the bicarbonate content is to some extent conserved in spite of phosphate retention. The vomiting is not the result of the reduction of the bicarbonate since vomiting is by no means a marked feature of diabetic acidosis.

For a more complete discussion of chlorides refer to pp. 174 and 175.

Phosphate: Inorganic phosphate increases as a result of renal insufficiency. As much as 10 mg./100 ml. are not uncommon in the plasma of uremic patients. The normal in adults is about 3 mg./100 ml. This accumulation is due to the changes in the lecithin content of the blood that accompany alterations in the level of the plasma proteins.

Sulfates: It is claimed by many authorities that inorganic sulfates may accumulate in the blood in renal insufficiency. Denis and Hobson,[45] in a study of 22 cases of nephritis and cardiorenal disease, found there was a marked increase over normal of the sodium and chloride in only 4 cases; the inorganic phosphate fraction was increased in 10 cases; whereas the inorganic sulfates were determined in only 17 cases, 11 of which showed increased values. Magnesium and potassium remained more or less constant while calcium was found to be decreased in 5 cases. Their studies demonstrated the specific selective activity of the kidney toward the various normal inorganic constituents of serum, somewhat similar to the selective activity which has been shown to exist for the various nonprotein constituents of blood.

It is probable that sodium and chloride, like creatinine, are excreted with great ease and, even in the case of badly damaged kidneys, retention of these elements seldom occurs. On the other hand, the sulfate ion is apparently excreted with difficulty so that in kidney insufficiency the concentration of this fraction may increase enormously, in some cases to 3000% above the normal value. In this respect the sulfate ion may be compared with the uric acid fraction, although the percentage increases observed are far above any concentrations of uric acid so far observed.

Relationship between uremia and blood chemical findings: It is generally believed that uremia is the direct result of accumulation of urea and kindred substances in the blood. Bright is responsible for this theory. In his Goulstonian lecture, delivered in 1833, he said: "What seems in some way to account for the general derangement and suffering of the constitution is the fact that urea becomes demonstrable in the circulating mass, and the blood becomes impregnated with that substance." Over 100 yr. after this statement was made this conception of the significance of urea retention has been amplified. We know now that urea is but one of the nitrogenous bodies that accumulate in the blood in nephritis and have learned that a similar retention occurs in the case of certain nonnitrogenous substances. This aspect of nephritis was given a fresh impetus at the beginning of the present century by the work of Widal and Javal and by that of Achard. Bennett[46] in his first Goulstonian lecture in 1928 in discussing "Some problems of nephritis" states that although the retention of nitrogenous substances in the blood is an invaluable aid in the diagnosis of renal failure, and although one may appreciate the fact that high nitrogen figures may become of great prognostic importance, one must also realize that nitrogen retention by itself offers no explanation of the actual phenomena of uremia. In summarizing the chief reasons against nitrogen retention as the cause of uremia, Bennett gives the following:

"1. The administration of urea and kindred substances to animals does not produce uremia.

"2. Conditions such as cystic disease of the kidney or prostatic obstruction often persist for years with marked nitrogen retention but no evidence of uremia.

"3. Cases of uremia without nitrogen retention are occasionally observed."*

The retention of nitrogenous substances in the blood is marked in renal failure, according to Bennett, largely because they are quantitatively among the more important substances which the kidney is normally called upon to excrete; but evidence accumulates to show that other chemical substances will show increased concentration in the blood, although to a lesser degree. In a very interesting case of cystic disease of the kidneys which Bennett was enabled to study up to the very last moment of life (ending in uremia) the blood analyses were in full agreement with the findings of most modern authorities; the actual figures in the terminal stage are given in Table 366.

Table 366. *Terminal phosphorus and chloride values in a case of cystic disease of the kidneys*

	Blood phosphorus (normal, 2-3)	*Whole blood chlorides (normal, about 85 mEq./L.)*
Nov. 2	6.3	86
Nov. 4	6.2	96
Nov. 5	8.3	97
Nov. 6	8.5	94

Bennett calls attention to the curious diazo reaction, first described by Andrews, given by the blood and cerebrospinal fluid in cases of uremia. It has been shown by Blotner and Fitz[47] to appear rapidly in dogs after nephrectomy, and, although its significance is still obscure, it seems very possible that this again is an example of the retention and concentration in the blood of some substance that the normal kidney would excrete.

Bennett in his lecture called attention to the outstanding features of similarity in cases of advanced renal destruction and cases of pyloric obstruction with tetany. He showed that in progressive renal disease there is found the following:

Acid accumulation in blood	Nitrogen retention
Acidosis	Calcium reduction
Phosphorus retention	Twitchings and convulsions

He found the following in nonmalignant pyloric obstruction:

Acid loss from vomiting	Nitrogen retention
Alkalosis	Calcium reduction
Chloride loss	Tetany

He called attention to the literature describing cases of pyloric or upper intestinal obstruction, in which routine histologic examination of kidneys removed at autopsy showed entirely unsuspected changes of a markedly degenerative nature in the renal tubules. These changes were of the type seen in cases of mercurial poisoning. It is evident that not only will renal damage produce profound changes in the blood chemistry but also that chemical changes in the blood may themselves exert a destructive effect upon the kidney substance.

Bennett has given full credit to the information obtained from biological chemists in elucidating facts concerning abnormal physiology of the kidney, but the fact remains that the phenomena of uremia are not altogether explained by the accumulation of blood chemical products in kidney degeneration.

TESTS FOR KIDNEY FUNCTION

There are a number of tests of kidney function, each group dependent upon certain physiologic principles. According to Belt[48] the requirements of a test of renal function may be summarized as follows:

1. It should be easy for the physician to apply and for the patient to carry out.
2. It should be easy of interpretation.
3. It should be carried out through the agency of a nondeleterious substance, preferably one with which the kidney is regularly called upon to deal.
4. The substance used should be one that is not influenced by any other organ in the body.
5. The test should reveal slight impairment and yield an answer in per cent of normal, ranging uniformly through all changes from

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*From Bennett, T. I.: Lancet 1:535, 1928.

slight impairment of function and structure to a total loss of kidney function.

6. It should reveal the nature of anatomic change occurring within the kidney.

7. The test applied in a chronologic series should have a prognostic value, indicating further degeneration or recovery.

Belt states further that no single test encompasses all of these criteria, that although the urea clearance test is constant and exact and covers the greatest range of function, it has the disadvantage of requiring technical aid in the form of chemical estimates of urea in both blood and urine. The range of the phenolsulfonphthalein test is much more restricted, being insensitive to slight failure in function and diminishing to the zero point long before the kidneys have approached a level of function in which life is in danger. As pointed out by Frothingham and Dennis, it indicates the function for the moment and not over an extended period of time. Its greatest value, which accounts for its popularity, is the fact that the laboratory procedures involved require the minimum of effort.

Dilution and concentration tests give the earliest warning of an impending failure of renal structure. Belt further emphasizes that the qualitative tests for albumin in the urine are very delicate and reveal quantities of this substance smaller than are pathologically significant. The xanthoproteic test gives a warning highly significant for judging danger to life.

Urea clearance test

For full details of the technic see p. 74. This test depends upon the excretion of a natural metabolite as it occurs in the body without artificial administration. For full data on the significance on the urea clearance figures see p. 74. In general, a reading of 5% shows that uremia is near at hand. The patient is still able to be up and about with readings of 10%. Twenty per cent is recognized as a dangerously low level although at this level an existing edema may clear and a

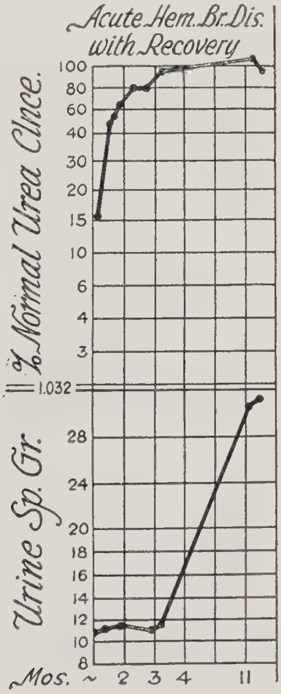

Fig. 675

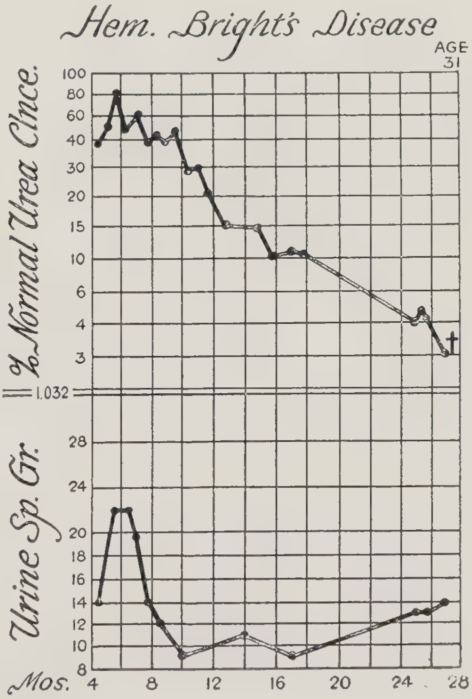

Fig. 676

Fig. 675. Recovery case of acute hemorrhagic nephritis. Changes of urea clearance and of urine specific gravity in Addis concentration test. Note that 3 mo. after onset clearance had returned to full normal value, whereas specific gravity test showed no improvement. Eleven months after onset both tests normal. (Courtesy Alving and Van Slyke.)

Fig. 676. Case of acute hemorrhagic nephritis with temporary improvement, followed by lapse into progressing chronic nephritis with eventual death in uremia. (Courtesy Alving and Van Slyke.)

sense of improvement in physical welfare is noted by the patient. At 52-56% the phenolsulfonphthalein test begins to be impaired. Readings of 70% or above indicate normal function. High readings indicate hyperfunction.

Sources of error in this test occur through inaccuracy in timing urine collections and incomplete emptying of the bladder.

The great value of this test is demonstrated in 2 charts reproduced from Van Slyke.[49] In explaining these charts Van Slyke stated that in Case 1 (Fig. 675) the clearance was first noted 1 mo. after onset, at 15% of average normal. Then during 2 mo. it rose to 100%. The specific gravity, which was very low at 1.010, showed no rise at all during these 2 mo. Improvement in this patient was genuine and was followed by complete recovery. The specific gravity figures 6 mo. after discharge were also found to be normal. For some time after the second month an abnormality remained in the kidneys which, although it did not interfere with urea excretion, was nevertheless indicated by the low specific gravity values that the concentration test continued to yield.

In Case 2 (Fig. 676) during the first 7 mo. the specific gravity test followed the urea clearance more or less closely. However, between the seventh and tenth months the specific gravity fell to 1.010, which is nearly the minimum reached in terminal nephritis. At this time the urea clearance still showed 30% of normal function. During the subsequent 20 mo., although the clearance gradually approached the uremia level at 5% of normal, the specific gravity showed no further fall.

Dye test of renal function

A number of different substances have been suggested for the so-called dye test of kidney function. The first dye used was methylene blue, which was introduced by Achard and Castaigne[50] in 1897. Later, indigo carmine was used by Heidenhain in his investigation of the physiology of the kidney. Later on, in 1903, Voelcker and Joseph suggested the use of the dye for the testing of renal function. Twenty ml. of a 0.4% solution were injected into the muscles of the gluteal region. Normally the urine became greenish blue 10-15 min. after the injection. Our experience with indigo carmine has been limited to the separate determination of the functions of the kidney when a cystoscope is introduced into the bladder and the appearance of colored urine trickling down from each side noted. This was termed chromocystoscopy. Rosaniline was introduced by Lepine[51] in 1898. One ml. of a 1% solution was injected subcutaneously. Geraghty and Rowntree[52] deserve credit for introducing the phenolsulfonphthalein test for estimating renal function.

Phenolsulfonphthalein test (PSP) of kidney function

The phenolsulfonphthalein test is frequently used in medical and surgical diagnoses. It has a definite field of usefulness but cannot be relied upon to the exclusion of other methods of testing kidney function. A low phenolsulfonphthalein output is very suggestive of renal injury. In surgical cases, in connection with operability or operative risk, the test is quite useful.

Reagents:

1. Ampules of phenolsulfonphthalein.*
    - (a) Dissolve 0.6 gm. phenolsulfonphthalein in 0.84 ml. 2N sodium hydroxide in 0.75% sodium chloride solution.
    - (b) Dilute to 100 ml. with 0.75% sodium chloride solution.
      This is the monosodium or acid salt, which is slightly irritant locally when injected.
    - (c) Add 2 drops 2N sodium hydroxide. Solution should change to a Bordeaux red. This solution is nonirritant.
    - (d) Ampule in 1.5 ml. quantities, seal hermetically, and sterilize.
    - (e) One ml. of this dye is injected intramuscularly.
2. Standard solution of phenolsulfonphthalein (100 ml. contains 0.6 mg. phenolsulfonphthalein).
    - (a) Add 1 ml. phenolsulfonphthalein solution (reagent 1d or commercial ampule of PSP) to 10 ml. 10% sodium hydroxide.
    - (b) Dilute to 1000 ml. with distilled water. This represents the 100% standard.
    - (c) Dilutions of the 100% standard should be made to represent 90%, 80%, etc., if desired.
3. Sodium hydroxide, 10%.
    - (a) Dissolve 10 gm. NaOH in enough distilled water to make 100 ml. Keep tightly stoppered.

Procedure for IM administration:

1. Instruct the patient to omit breakfast before reporting to the laboratory in the morning.
2. Give the patient 600-800 ml. water about 30 min. prior to the injection.
3. 30 min. later inject 1 ml. sterile PSP solution.
4. Allow 10 min. for the beginning of the excretion of the drug.
5. 1 hr. 10 min. after the injection collect the urine in a bottle labeled "No. 1." Collect the entire specimen.

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*Ampules containing 6 mg. PSP/ml., sterile, may be obtained commercially.

6. 1 hr. after this instruct the patient to collect the second specimen in a bottle labeled "No. 2." In patients with obstruction to the flow of urine, the urine is drawn off at the end of each hour. Other patients may simply be allowed to urinate at hourly periods.
7. To the entire first-hour specimen in bottle No. 1 add 10 ml. 10% sodium hydroxide.
8. Rinse the mixture into a 1000 ml. graduated cylinder with a few ml. water.
9. Dilute to 500, 750, or 1000 ml., depending on the depth of color. The color must be lighter than the color of the standard soluion if visual colorimetry is used.
10. Read in the colorimeter.
11. Calculate the results of the first specimen.
12. Carry out the same procedure with the second specimen and calculate the results. Report the entire amount of dye excreted.

Procedure for IV administration:

1. Steps 1 and 2 above.
2. 30 min. later inject 1 ml. sterile PSP solution intravenously.
3. Collect urine specimens at 15, 30, 60, and 120 min.
4. Measure PSP specimens as described above.

Calculation*:

$$\frac{\text{Reading of standard}}{\text{Reading of unknown}} \times \frac{\text{Dilution of unknown}}{\text{Dilution of standard}} = \%\text{ of dye excreted}$$

Example: Reading of standard is 15; reading of unknown is 20; dilution of standard is 1000; dilution of unknown is 750.

$$\frac{15}{20} \times \frac{750}{1000} = 56\tfrac{1}{4}\%$$

Photoelectric colorimeter: Wavelength 520 mμ.

$$\frac{OD_u}{OD_s} \times \frac{\text{mg. standard}}{6} \times 100 = \%\text{ of dye excreted}$$

Suppose 56¼% of dye were eliminated the first hour and 30% the second hour. Report as follows:

First hour	56¼%
Second hour	30 %
Total dye eliminated	86¼%

Normally about 60-75% of the dye should be excreted in 2 hr.

Many prefer to collect specimens at 15 min., 30 min., 1 hr., and 2 hr. Normal ranges for these time intervals are 28-51, 13-24, 9-17, and 3-10% excretion, respectively.

This is a very important test in connection with the estimation of kidney function. Normal kidneys excrete 60-75% of this dye in the 2 hr. period allowed for the test. A fall to 40% indicates impaired function. Reduction to 20 or 30% is the rule in well-marked cases of nephritis, whereas excretions of less than 10% may be interpreted as very serious. The exact interpretation of the findings of the phenolsulfonphthalein test, however, is that it indicates a pathologic condition of the kidneys for the moment and not over an extended period of time. In order to obtain a true estimate of the condition of the kidneys over an extended period of time, one must resort to blood chemical methods.

According to the work of Frothingham and Dennis, it is known that the phenolsulfonphthalein test may be normal or may be increased in acute glomerulonephritis. In certain other cases of acute nephritis there may be an extremely low excretion, almost to zero. These figures are based largely upon experimentally induced nephritis, induced by the injection of uranium nitrate. In chronic nephritis in clinical practice we have known the phenolsulfonphthalein output to be between 70 and 85% and still there was marked damage to kidney function. It is a clinical fact that low excretion from 10 to 15% is the rule in cardiac decompensation with chronic passive congestion of the kidneys. This rises rapidly when the congestion is relieved by improved heart action. In chronic nephritis, although the estimation of this test is important, it sometimes presents inexplicable variations.

LaMotte phenolsulfonphthalein apparatus—roulette type

Phenolsulfonphthalein is injected and the specimen collected as in the test described on p. 1892. The method of reading the test, in which advantage is taken of the chemical indicator properties of phenolsulfonphthalein, may be termed the bicolor method. This completely eliminates the effects of color and turbidity of the specimen in making the color comparison.

Concentration and dilution tests

As indicated before, the concentration tests of urine are mainly for the purpose of determining the functional ability of the tubules.

Mosenthal 2 hr. specific gravity test

This is a simple method of ascertaining the concentration power of the kidneys. The specific gravity is taken on 2 hr. samples of day urine, and the amount of the night urine is then measured. For healthy individuals this amount is 400 ml. or less, while the specific gravity is 1.018 or higher. In the 2 hr. samples of the day urine the specific gravity fluctuates 10 points or more from the highest to the lowest point.

Impairment of kidney function is noted by an increase in the night urine, that is, it goes above 400 ml., while the specific gravity is low, around 1.015. Lower specific gravity is seen with very little variation in the 2 hr. specimens of day urine. The specific gravity is fixed and does not change.

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*Comparators are available.

The patient must be directed not to take any liquids or foods after supper or in the morning until the morning urine is voided. He is permitted to take breakfast or noonday meal of an ordinary type but must take no liquids or foods between meals.

This test is extremely valuable in nephritis because it tells some general points about kidney efficiency and gives data on the water excretion.

Interference with the transudative power of the glomeruli and the secretory property of the tubules can be ascertained by observing the disproportion between fluid intake and fluid elimination. Use the **water test of Volhard.**

Patient must remain in bed during the test. Give 1500 ml. water in the morning on an empty stomach. Collect the urine every hour for 4 hr. and later every 2 hr. After 4 hr. the patient is on a dry diet. Normally most of the consumed liquid is eliminated during the 4 hr.; small amounts are eliminated during the latter part of the day. In the second and third hours after water consumption specific gravity varies between 1.001 and 1.003 rising later in the day to 1.027.

Deviations from the normal: If in the first 4 or 5 hr. more urine is eliminated than water consumed, e.g., 1600 ml., with a specific gravity of about 1.008 to 1.010, but not very low (isothenuria), it indicates severe kidney damage. Some patients may eliminate less fluid than was consumed, as in cases approaching edema, such as hypothyroidism, cardiac decompensation, and the later stages of nephrosis.

Fishberg concentration test

This is a simpler method than that of Mosenthal. It is fully described in the classic volume of A. M. Fishberg, *Hypertension and nephritis.* On the evening before the test the patient takes his supper at 6 P.M. This meal should contain a large amount of protein, but the fluid intake should not be more than 200 ml. No food or drink may be taken after this until the test is finished. The urine should be voided before retiring and should be discarded. The first specimen saved is the morning urine. The patient remains in bed and another specimen is collected 1 hr. after the first in another container. A third specimen is collected in still another container 2 hr. after the first. The specific gravity of each of the 3 specimens is taken. Under normal conditions the specific gravity should be over 1.024 in at least one specimen.

This test is based upon the fact that concentration is the most essential function of the normal kidney, especially the tubular portions, and that any decrease in this quality is a prime indication of renal impairment. Accordingly, under the conditions of this test the specific gravity, which indicates the concentration of urinary constituents, should be increased if the kidney function is normal, for the reason that no fluid is being ingested during the performance of the test. At the same time, if the kidney function is materially disturbed, the specific gravity will be lower than usual. Accordingly, the figure 1.024 is fixed as the minimum level for normal urine. In cases of uremia the specific gravity figures are surprisingly lower, sometimes as low as 1.010.

The test cannot be used when there are conditions of uremia, but it is very useful in early cases of renal impairment, especially to indicate the impairment of the tubular portions of the kidney.

Sodeman-Engelhardt concentration test[53–56]*

The renal concentration test employing posterior pituitary extract is based upon the renal action of this substance, which has been shown to inhibit water diuresis as well as increase the chloride excretion. It reduces urinary volume and increases the concentration of solids in the urine. This action takes place in spite of the ingestion of water, so that previous preparation of the patient by fluid restriction is unnecessary.

In practice the following procedure is carried out: The unprepared subject empties the bladder and the specimen is saved. Immediately 10 units (1 ml. obstetric or 0.5 ml. surgical) posterior pituitary extract is given subcutaneously. The subject receives nothing by mouth until the test is completed. Specimens are obtained at the end of the first and second hours. The specific gravities of the 3 specimens are then taken, and the usual corrections for albumin and temperature are made, if necessary.

In normal individuals at least one of the specimens will show a specific gravity of 1.022

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*Courtesy of the originators of the test, Drs. W. A. Sodeman and H. T. Engelhardt of New Orleans, La.

or more. Urinary volumes may be small, but inexpensive and accurate urinometers requiring only 4-6 ml. urine are available.

The dosage of posterior pituitary extract used does not affect the blood pressure. Hypertension is not a contraindication to the test. The extract causes coronary constriction and should be used with caution in angina pectoris. Acute coronary occlusion, sensitization, and late pregnancy are contraindications to its use.

The test is simple, requires only minimal cooperation by the patient, particularly as far as fluid restriction is concerned, and can be carried out immediately, without preparation of the patient, at any time of day. It is particularly indicated in edematous patients when other concentration tests are inaccurate because of diuresis. Furthermore, it is of value in surgical patients when restriction of fluids is contraindicated.

Volhard-Fahr concentration and dilution tests[48]

Concentration procedure:

1. Allow no fluids from the evening before the test until the test is completed and no food between meals. All 3 meals must be without liquids and without liquid food.
2. 8 A.M. Have patient empty bladder. Thereafter collect urine in separate containers every 3 hr. until 8 P.M.; that is, at 11 A.M., 2 P.M., 5 P.M., and 8 P.M., and collect all urine from 8 P.M. until 8 A.M. the next morning in 1 container.
3. Note the quantity and specific gravity of each 3 hr. sample and of the 12 hr. sample and plot as a curve. Normally the specific gravity of at least one sample should be 1.030 or at least 1.025.

Dilution procedure:

1. Have the patient omit breakfast on the day of the test. For lunch and dinner give the patient the diet to which he has been accustomed.
2. 8 A.M. Have patient empty bladder and give him 1500 ml. water.
3. Collect urine in separate containers at 8:30, 9, 9:30, 10, 10:30, 11, 11:30 A.M., and 12 noon, 8 specimens in all.
4. Collect all the urine from 12 noon to 8 A.M. the next morning in one container.
5. Note the quantity and specific gravity of each sample and plot as a curve. Normally the total quantity voided should be 80-120% of the intake (1200-1800 ml.). The specific gravity of at least one sample should be as low as 1.003.

Isberg-Newburgh 18 hr. concentration test of kidney function

Isberg and Newburgh[57] described a simplified concentration test of kidney function based on the 38 hr. concentration test of Lashmet and Newburgh.[58] The test is performed over a period of only 18 hr. and does not require the use of a special diet. It is particularly convenient for use on ambulatory patients. A simple, rapid, and accurate method for quantitative determination of proteinuria may also be made. This test is based on the comparison of the turbidity of urine, in which protein has been precipitated by 2% sulfosalicylic acid, with the turbidity of a permanent, standard suspension of inorganic substance.

Procedure: The subject finishes his usual supper by 6 P.M. He then has nothing to eat or drink until noon the following day; he may carry on ordinary activity during this time. Collect urine specimens at 8 A.M., 10 A.M., and 12 noon, with complete emptying of the bladder each time. Determine the specific gravity of each specimen with the urine at room temperature and with an ordinary urinometer which has been checked with distilled water.

Test the most concentrated specimen for the presence of protein, and, if present, determine the amount quantitatively in order to correct the specific gravity when necessary. For every 1% of protein present in the urine, subtract 0.003 from the observed specific gravity; any proteinuria less than 0.3% does not need to be considered. Thus the specific gravity of most urines will not require correction.

Isberg and Newburgh have further simplified the easy and accurate method of Lashmet and Newburgh[58] for quantitative measurement of proteinuria.

Reagents:

1. Standard turbidity suspensions.
    - (a) Stock suspension. To 200 ml. of distilled water in a 500 ml. volumetric flask add 50 ml. N/10 sodium hydroxide solution and 8 gm. copper sulfate, hydrous. Dilute to 500 ml. with distilled water. Shake vigorously to ensure uniform turbidity.
2. Standard tube.
    - (a) Shake the stock solution thoroughly and transfer exactly 2 ml. to a test tube, using a pipet.
    - (b) Add 23 ml. distilled water.
    - (c) This standard tube can be made permanent by drawing out its open end and sealing in a flame. **Before using, invert the standard tube several times to obtain uniform turbidity.**
3. Sulfosalicylic acid, 2%.
    - (a) Dissolve 2 gm. sulfosalicylic acid in enough distilled water to make 100 ml. solution. This reagent may turn pink on standing. Such solutions should be discarded, for the pink cast interferes with accurate comparison with the standard.

Procedure:

1. Use graduated 15 ml. conical centrifuge tubes. Place mark U at 1.66 ml. for tube 1, at 0.83 ml. for tube 2, and at 0.5 ml. for tube 3. Place mark R at 12.5 ml. on each tube. Tube

1 represents 0.3% protein, tube 2, 0.6%, and tube 3, 1% protein. (See Fig. 677.)

2. To 1 ml. urine, add 9 ml. distilled water, making a dilution of 1:10. Add the diluted urine to U mark of tube 1 and then add 2% sulfosalicylic acid up to mark R. Mix thoroughly and allow to stand for 3 min. If the resulting turbidity matches that of the **standard tube**, the urine contains 0.3% protein; the specific gravity of the urine is then corrected by subtracting 0.001. If the turbidity is less than that of the standard tube, no correction of the observed specific gravity is necessary.
3. If the turbidity of tube 1 is greater than that of the standard tube, add the urine diluted 1:10 to U mark of tube 2 and then add 2% sulfosalicylic acid to R mark. Mix thoroughly and allow to stand; if the turbidity matches that of the standard tube, the urine contains 0.6% protein. The observed specific gravity then is corrected by subtracting 0.002. If the turbidity is greater than that of the standard, add the diluted urine to U mark of tube 3 and sulfosalicylic acid to the R mark. If the turbidity resulting in tube 3 matches that of the standard tube, the urine contains 1% protein. The observed specific gravity is then corrected by subtracting 0.003. Urine containing more than 1% protein should be diluted 1:20, and the foregoing procedure with the 3 tubes repeated.

A maximum specific gravity of 1.025 is considered the lower limit of **normal** for this 18 hr. renal concentration test.

Of 200 cases of essential hypertension, 124 cases, or 62%, showed impaired concentrating ability. The concentration test was found to be more sensitive than the urea clearance test in detecting early kidney damage in essential hypertension. These authors also found that in each of 8 cases of glomerulonephritis, both subacute and chronic, there was impaired concentrating ability. The maximum specific gravity in these cases ranged from 1.010 to 1.020.

Addis urea concentration test

This test is for ambulant patients—not for patients with edema or very high diastolic pressure or failing heart or in the late stages of Bright's disease.

Procedure:

1. Instruct the patient to abstain from fluids of all sorts after breakfast on one day until he arises from bed on the following day and tell him to collect a 12 hr. specimen of night urine, voided directly into a wide-mouthed bottle. It must be made plain that the urine passed at the beginning of the 12 hr. period is to be discarded and only the urine saved

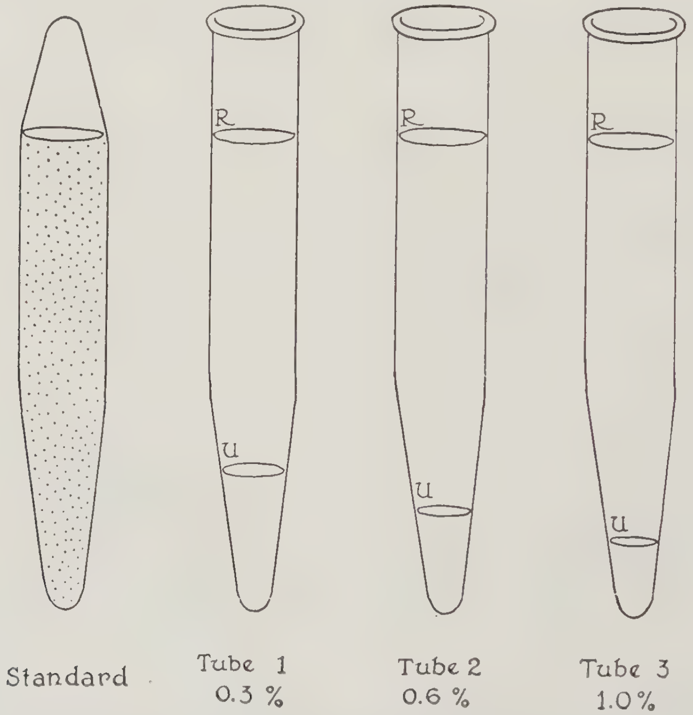

Fig. 677. Set of tubes for rapid quantitative determination of proteinuria. **Tube 1: U** mark **is** at 1.66 ml. = 0.3% protein. **Tube 2: U** mark is at 0.83 ml. = 0.6% protein. **Tube 3: U** mark is at 0.5 ml. = 1% protein. The **R** mark is at 12.5 ml. (From Isberg, E. M., and Newburgh, L. H.: Am. J. M. Sc. **211**:701, 1946.)

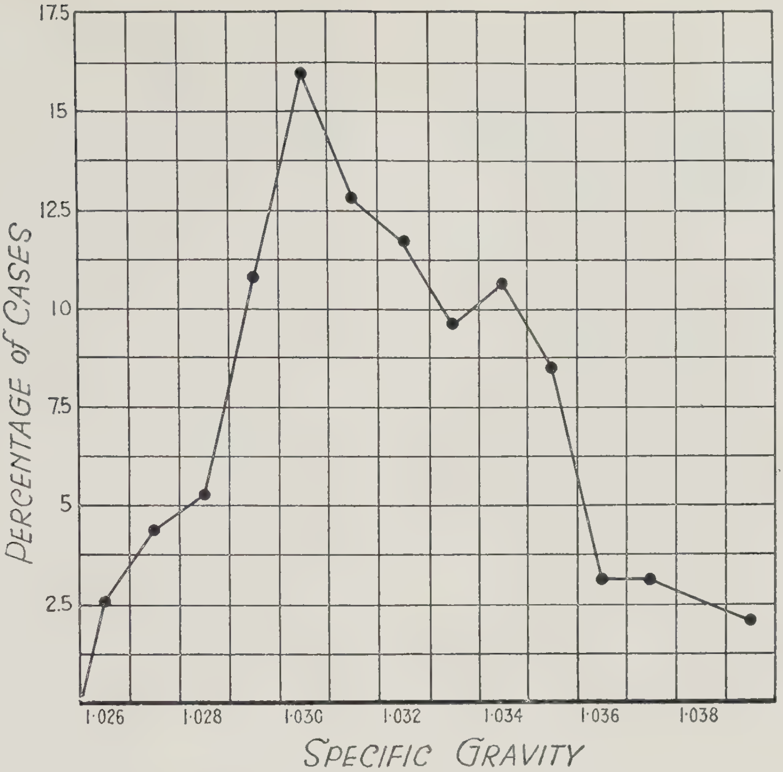

Fig. 678. Percentage frequency distribution of specific gravity of the night urine in normal individuals after restriction of fluids. (From Addis, T., and Shevky, M. C.: Arch. Int. Med. **30**:559, 1922.)

that has been formed by the kidney during the 12 hr. of the night.

2. Instruct patient that usual diet may be taken but that water, tea, coffee, milk, soup, or any other fluids must be avoided.
3. Measure the specific gravity.

In standardizing this test Addis made 94 observations on 75 normal persons. The average specific gravity was 1.032; the standard deviation ± 0.00281, and the variability 8.8%. Fig. 678 shows Addis' chart revealing the percentage frequency distribution of specific gravity.

Addis count of urinary sediment

The Addis count is a procedure to make the differentiation of the formed elements of the sediment of the urine more accurate. It was described by Thomas Addis in a communication entitled, "A clinical classification of Bright's disease."[60]

Procedure: Secure the intelligent cooperation of the patient by written instructions given to him and spending some time with him to be sure he understands what is required. Let him take his usual breakfast next morning, including coffee, tea, or milk. Thereafter, he must abstain from all fluids during the day and night until the urine has been collected on the following morning. Fluids are defined as all substances that can be poured from one vessel into another. In other respects his usual diet is left unaltered, and he is specifically instructed not to take more fruit than is customary, even though he may become thirsty. The collection of urine may extend from any convenient hour in the evening until the patient rises from bed in the morning, say from 6 P.M. to 6 A.M. Be sure that the bladder is completely empty at the beginning and at the end of the collection. Note the time exactly. Unless there is a considerable volume of urine in the bladder, it often does not completely empty itself. The patient is therefore asked not to urinate during the afternoon of the day on which the collection is begun and to void the total night urine when the collection is completed. The time at which the collection is begun and the time at which it is ended must be written by the patient on a label on the bottle into which the urine is voided.

Remember that the urine passed at the beginning must be discarded and not collected at all. The urine is passed into a wide-necked bottle which has been thoroughly cleaned, washed with distilled water, dried in the inverted position, and finally rinsed with a clean solution of formaldehyde, which is allowed to drain out for a short time before the bottle is closed with a clean rubber stopper. In women, catheterization is essential for this procedure. The nurse must take pains to be sure that the bladder is really emptied, though even the possibility of an incomplete collection must be remembered.

The patient returns the next morning, early, with the bottle of urine. It is almost always in a condition suitable for examination. If it is neutral or alkaline, there is a precipitate of phosphates. If the salt concentration is not too low, hyaline casts may still be found after the urine is cleared by the addition of concentrated acetic acid; but, since it is impossible to be certain that partial solution may not have occurred, cast counts are never made on such specimens, although the number of white blood cells and small round epithelial cells may be determined. If there is a precipitate of urates, the precipitate should be dissolved by placing the bottle in hot water.

Measure the volume of urine and return it to the bottle in which it was collected, reinsert the rubber stopper, and mix thoroughly by repeatedly inverting the bottle.

Immediately thereafter, transfer 10 ml. to a special graduated centrifuge tube with a diameter so narrow toward the tip that an accurate reading may be made.*

Centrifuge the tube for 5 min. at 1800 r.p.m.

In normal persons there is a deposit in the centrifuge tube of about 0.2 ml. of what seems to be mucus. In the initial stage of the condition described as hemorrhagic Bright's disease (glomerular disease), if the renal lesion is pronounced, there is a large amount of brown precipitate. In the active stage there are diminishing degrees of the brown color until in some cases it is imperceptible and the sediment is white. In the latent stage there is often no appreciable deviation from what is observed in normal urine. In the terminal stage there is no mucus; therefore the cells and casts form a closely packed white deposit at the extreme tip, giving a characteristic appearance.

In degenerative Bright's disease (disease of the tubules) no brown color is ever to be seen, and the amount of cloudy white deposit varies with the number of casts and epithelial cells. In arteriosclerotic Bright's disease (disease of the blood vessels) there is simply the colorless mucus as obtained from the urine of normal persons.

Decant the supernatant urine and remove part of the remainder with a pipet until it is reduced to a volume the magnitude of which will vary with the amount and nature of the deposit. In urines with appreciable concentration of protein a precipitate appears at the surface of the urine as its meniscus approaches the narrowing tip of the tube. In such cases add a few drops of 1% sodium chloride solution and again draw the fluid off until a convenient volume has been reached.

All the casts and cells from the 10 ml. sample of the urine are concentrated in the volume that has been selected. Draw up and blow out the fluid with a capillary pipet, thus securing an even distribution of these elements. Add a drop into each side of a blood-counting chamber and count the number of casts under the low-power field over all the ruled areas. The area counted depends on the concentration of casts. In the urine of normal persons there are very few casts, and 10 drops are usually examined. When casts are numerous, the deposit is diluted 1:5, and only 2 drops are counted over a known area. The cells are counted under a high dry objective and usually over unit areas of 0.1 sq.mm. The differential cast count is made while the casts are being counted.

In the latent stage of hemorrhagic Bright's disease, when the number of casts is small, it is best to obtain a more concentrated mixture by centrifuging urine pipetted from the bottom of the collecting bottle after it has stood still for an hour or more.

Calculation: Since the chamber is 1/10 mm. deep, the volume contained over the total ruled area is 0.9 c.mm., or 0.0009 ml. If 10 areas are examined, the volume of urine in which casts are counted is 0.009 ml. Suppose that in this volume 90 casts are found and that the volume of urine or salt solution in which the sediment was mixed was 0.9 ml. In that case $90 \times \frac{0.9}{0.009}$, which is 9000, the number of casts in 0.9 ml. But all the casts in 10 ml. urine were concentrated in that 0.9 ml. volume—so 9000 is the number of casts in 10 ml. urine. If the urine volume for 12 hr. was 300 ml., the number of casts in 12 hr. is $9000 \times \frac{300}{10}$, or 270,000. The same method of calculation is used in the cell counts. The general formula is as follows:

$$\text{No. counted} \times \frac{\text{Vol. in ml. in which sediment was mixed}}{\text{Vol. in ml. in which count was made}} \times \frac{\text{Vol. of urine in ml. per 12 hr.}}{10} = \text{No. in 12 hr. urine}$$

Addis count in children: Boyle et al.[61] investigated the usefulness of the Addis count in estimating recovery of children from post-infectious nephritis. The Addis count, described by Addis,[60] was based upon the idea that the type of renal pathology might be predicted from study of the sediment. In 1926[62] he published what he considered to be normal figures for the various constituents, particularly red blood cells, leukocytes, epithelial cells, and casts. Goldring[63, 64] made studies of the sediment of clinically well adults and of adults with nephritis, rheumatic heart disease, subacute bacterial endocarditis, and pneumonia. His averages for normal adults were higher than those of Addis. Naeraa[65] reported figures for normal adults higher than those of Addis and lower than those of Goldring. He claimed that the number of red blood cells increases with the age of the individual.

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*Convenient and accurately made tubes are furnished by Arthur Thomas & Co., Philadelphia, Pa.

The results of the examination of the urinary sediments of the patients who were investigated by Addis fall into classifications in consonance with the classification of Bright's disease used by him. He calls the first division hemorrhagic Bright's disease; the second, degenerative Bright's disease; and the third, arteriosclerotic Bright's disease.

Addis count in hemorrhagic Bright's disease: This is glomerulonephritis. In this condition the urine contains so many red blood cells that it has a mahogany brown appearance. The precipitate consists of lysed and distorted red blood cells, with a considerable admixture of pus cells and epithelial cells. Casts found in this cellular debris vary from lemon yellow to dark brown. Red blood cells are found. Blood casts are found which are pathognomonic of hemorrhagic Bright's disease. The symptomatology of this disease is quite variable, but there is usually a moderate increase in diastolic pressure and often a slight, generalized edema.

The active stage follows the initial stage, or it may be the result of an exacerbation of the continuing streptococcal infection in a patient with latent hemorrhagic Bright's disease. It is accompanied by an increase in the number of casts and in the rate of protein excretion and by the appearance of fatty casts and fat droplets on all the hyaline casts. If there is no increase in blood pressure, it is only by the large number of red blood cells and the finding of an occasional blood cast that the condition can be distinguished from what Volhard and Fahr called "genuine nephrosis." With the disappearance of the inflammation the lesion may become latent and heal; with its continuance it will progress, either directly or after an interval of latency, to the terminal stage.

As the terminal stage approaches, there is a gradual change in the nature of the urinary sediment. As one after another of the inflamed glomeruli become completely disabled and fibrosed so that urine no longer flows down their tubules, fewer red blood cells and pus cells come from the shrunken kidney, and blood casts are hard to find. When the amount of secreting tissue is decreased to less than ⅓ of its original amount, the urea concentration of the blood begins to rise; and, as the kidney grows smaller and smaller, the blood urea concentration rises higher and higher. All the casts are renal failure casts.

Addis count in degenerative Bright's disease: This second classification has been called degenerative Bright's disease because the most prominent feature of the sediment is the large number of epithelial cells in various stages of granular or fatty degeneration. It is impossible to determine whether they were originally epithelial or white blood cells. Addis counts them together.

Degenerative Bright's disease is not an entity but only a convenient term under which to group all those many pathologic conditions that may give rise to degenerative changes in the kidneys. They may be summarized under 6 divisions.

1. Certain cases of unknown etiology are called cryptic degenerative Bright's disease. These cases are usually seen only when edema has developed. Nothing is known of the initial stage.
2. There are patients with degenerative Bright's disease in whom the chemical constitution of the causative agent is known, such as mercury, chromium, and uranium salts, as in jaundice and in some cases of malaria.
3. The unknown toxin derived from the fetus in the latter half of pregnancy produces a degenerative Bright's disease accompanied by hypertension.
4. Toxemia that may accompany any generalized infection is likely to produce a slight cloudy swelling of the kidney. This may take the form of desquamation of renal cells with many epithelial and granular casts and much protein.
5. Focal infections are not often associated with any but minor grades of renal degeneration, but some patients develop proteinuria and edema.
6. Mixed infections which are found in long-standing osteomyelitis with sinus formation tend to produce the waxy kidney.

Addis count in arteriosclerotic Bright's disease: This is the final classification of Addis. It occurs more commonly than any other form of Bright's disease. So far as the kidney is concerned, it is of negative character, for, when the diagnosis has been made, it is no longer necessary to consider the renal lesion as a factor in the management of the patient. These are the patients with hypertension who are often told that they are suffering from "chronic interstitial nephritis" and who consequently live in fear of death of a uremia that never comes. With examination of the urinary sediment by Addis' method, it is possible to construct a clear picture of the genesis, the rise and fall, and, if fibrosis advances, the terminal diminution in the activity of the renal lesion; the classification that grows out of such observations is an advance toward a purely etiologic classification, the classification that must always remain the one toward which we strive.

Xanthoproteic reaction

Volhard termed the xanthoproteic test a criterion for judging the danger to life of the kidney lesion. It was assumed by Beecher that the symptoms of uremia parallel the accumulation of products of intestinal putrefaction, such as phenol, phenol derivatives, paracresol, and oxy acids in the blood. These undetoxified products of intestinal decomposition accumulate in the blood and tissues because the detoxifying processes are no longer carried out completely in the wall of the intestine and the liver. The chromogen materials are retained in the skin, and these give the yellow color to the skin of uremics.

Procedure:

1. To 2-3 ml. of the solution to be tested add concentrated nitric acid.
2. Upon the addition of nitric acid a white precipitate forms which disappears; the solution turns yellow upon heating.
3. Cool and add ammonium, potassium, or sodium hydroxide. The color of the yellow solution deepens to orange.

This chemical reaction is due to the phenol group with which nitric acid forms nitro modifications. Hawk criticized the test as not satisfactory for use in urinary examinations because of the indeterminate color of the end point, thereby making it qualitative only.

CORRELATION OF RENAL FUNCTION TESTS

It is not often possible to correlate the various tests which are supposed to express kidney function in per cent of normal. As Belt[48] has summarized in his excellent review of this subject, readings taken at the same time in the same individual may be quite low in one type of test and definitely high in another. On the other hand, a very close correlation will be found to exist between different readings of the same test done on successive days. This discrepancy arises through the fact that each particular test must be an expression of a slightly different phase of kidney function. In order to give a graphic expression of these facts, Belt compiled a chart (Fig. 679) which

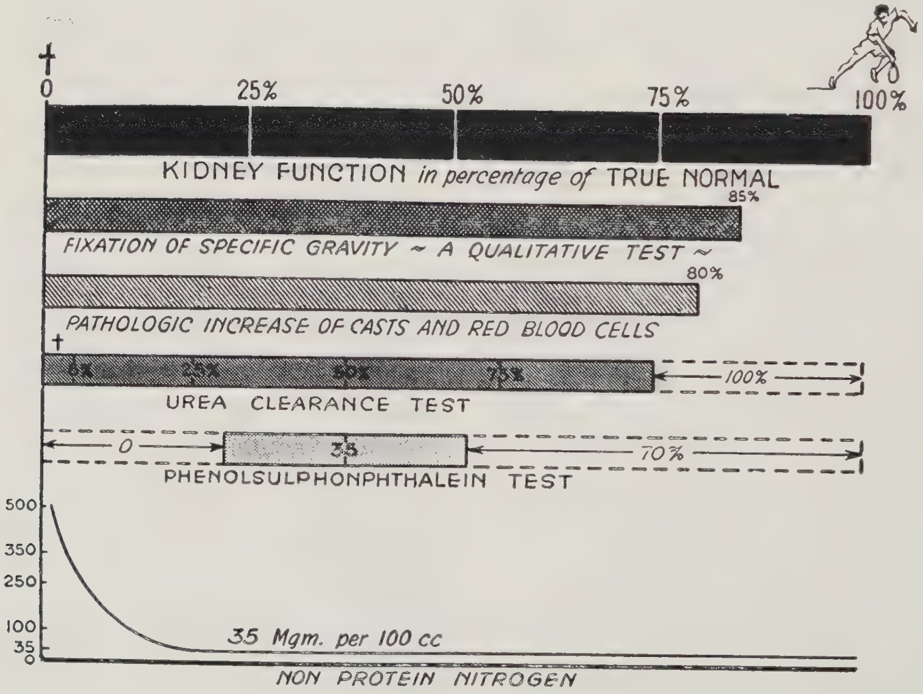

Fig. 679. Correlation of renal function tests. Solid bar at the top represents true kidney function. This is never completely measured by any test. The tests in current use are represented below by a series of shaded bars. The length of each indicates its range in comparison with the others and with the true normal. (From Belt, E.: In Cabot's modern urology, Philadelphia, 1936, Lea & Febiger.)

shows that the earliest sign of renal dysfunction is a fixation of specific gravity. This test is extremely sensitive in revealing slight renal damage, but it does not distinguish between a moderately advanced and a terminal stage of the disease. He states that an abnormal number of casts and red cells is the next most sensitive indication of disorder. This has a slightly quantitative significance when differential counts are compared from time to time. He calls attention to the fact that the urea clearance test has the widest range of all the tests attempting to express function in per cent of normal. It is not sensitive to changes less advanced than are represented by a loss of 20% of the kidneys' functional capacity but does permit readings on the low side of the scale all the way down to 5%.

Furthermore, the phenolsulfonphthalein test is less sensitive to early changes than is the urea clearance test. Zero readings are recorded by it when a wide margin of kidney function still exists.

The nonprotein nitrogen begins to be elevated as the phenolsulfonphthalein test approaches zero and is always high when the urea clearance test is 20% or below. In the absence of general metabolic factors which might influence the degree of elevation of the nonprotein nitrogen, its level above normal marks the diminution of secreting tissue toward zero.

Chapter 104

Interpretation of urinary findings

SAM FRANKEL

Chyluria

There are 2 kinds of chyluria: (1) the tropical form due to invasion of the blood and urinary tract by the *Wuchereria bancrofti* and (2) a variety that is not parasitic but is of unknown etiology. The disease is the result of lymphatic obstruction. The urine is of an opaque white color, closely resembling milk; under the microscope the turbidity seems to be caused by numerous minute granules, the fat being in molecular form. The only difference between lymph and chyle is that the latter contains a much larger percentage of fat.

The generally accepted theory of the cause of chyluria holds that there is a fistulous communication between the lymphatic and urinary tracts and presupposes a lymphatic obstruction somewhere between the small intestines and the thoracic duct. Chyluria occurs in the presence of thoracic duct obstruction only when the collateral lymphatic circulation is inadequate.

The lymphatic vessels of the kidney form 3 plexuses: one in the substance of the kidney, a second beneath its fibrous capsule, and a third in the perinephric fat. The second and third communicate freely with each other. The vessels from the plexus in the kidney substance converge to form 4-7 trunks which issue at the hilum. Here they are joined by vessels from the plexus under the capsule and, following the course of the renal vein, end in the lateral aortic glands. The perinephric plexus is drained directly into the upper lateral glands.

The route from the intestinal to the renal lymphatics has been the subject of much speculation. According to Magnus-Levy, the chyle must first go from the mesenteric lymph channels through the mesenteric lymph glands to the thoracic duct. Then, because of obstruction, there is a retrograde flow to the upper lumbar lymph glands which drain the renal lymphatics. To obtain such a retrograde flow an insufficiency of the valvular system of the lymphatic channels must be assumed. Many explanations have been offered for chyluria, but the theory of lymphatic block and direct urinary tract communication is probably the most sufficient for the present.

Symptoms: The symptoms of chyluria are mainly the milky appearance of the urine, which suddenly follows the partaking of a meal rich in fats. It may develop after a strain, or it may appear without any demonstrable cause. It has been noted during childbirth with subsequent cessation after delivery. It may be continuous or intermittent. In some cases it is present at all times; some have observed it in only the night urines, clearing as the patient became active during the day; still others noted chyle in the day urine only; again it was found only when the patient had been exercising vigorously; it may be influenced by posture.

Lipuria and marked **pyuria** may be confused with chyluria; in the former the fat is present in the droplet form although occasionally emulsified to the extent of a milky color. Microscopic examination will differentiate the pyurias. The fat in the urine of chyluria is in molecular form and therefore presents no morphology. In such cases the presence or absence of fibrinogen, cholesterol, or lecithin will be of significance.

In chyluria the urine contains coagula, due to the large amount of fibrin. These clots may form in the bladder and be abundant enough to give rise to symptoms. The specific gravity is low, reaction is acid, and there is a large amount of albumin. Microscopically the clots show fibrin with a number of red cells embedded with small plates of hematoidin. There are many red cells and many small fat globules. Pus corpuscles and epithelia from the ureters and bladder are found. In the tropical variety one may see the embryonal forms of the filaria.

Two glasses of equal parts milk and cream should cause chyle to appear in a catheterized specimen of urine removed at the end of 1 hr. A second specimen should be taken at the end of 4 hr. If the test is negative, it should be repeated with the patient in the recumbent position. Since chyluria is intermittent, all the tests may be negative, but if injected perirenal lymphatics are present and the tests are repeated at intervals, chyluria will undoubtedly be found. Hampton kept his patient on Johns Hopkins Hospital diet, adding an extra amount of butter and giving 2 glasses of milk before each test. He catheterized the kidneys every hour for 4 hr. Chyle was present in large amounts in the first-hour specimen and had practically disappeared in the last collection.

Renal tuberculosis

In early cases of renal tuberculosis the urine may be practically normal in appearance, but it is frequently cloudy from pus. The volume may not be affected but is likely to be increased, the reaction is acid, and there are traces of albumin and a few renal cells. In advanced cases the urine is usually pale, cloudy, and alkaline, has an offensive odor, and is irritating to the bladder. In such cases albumin and pus are always present though frequently not abundant. The pus is generally intimately mixed with the urine and does not settle as quickly as does the pus of cystitis. Casts are seldom abundant and are obscured by the pus. Tubercle bacilli are nearly always present even when the pus is extremely slight and their detection is essential for the diagnosis. In some cases they may be found by appropriate staining of the sediment after prolonged centrifugation. In other cases they cannot be found, and it is necessary to inoculate guinea pigs to make the diagnosis.

Hirschberg[66] has discussed the demonstration of tubercle bacilli in the urine. The guinea pig inoculation method has been criticized because many guinea pigs contract tuberculosis spontaneously or have internal lesions which resemble those caused by the tubercle bacillus. Howell and Schultz[67] report an epizootic among guinea pigs due to a paratyphoid B bacillus which caused lesions closely resembling tubercles in the spleen and liver. Magath and Feldman[68] conclude that with reasonable care spontaneous tuberculous infection of guinea pigs can be avoided. Cummings[69] reported the incidence of spontaneous tuberculous infection in laboratory animals in Saranac, a tuberculosis center. Hirschberg attempted to make comparisons between the direct smear, the guinea pig inoculation, and culture methods for the demonstration of tubercle bacilli in the routine specimens received in the laboratory of a large hospital, reaching the conclusion that for the demonstration of tubercle bacilli in the various types of specimens the culture method gives a greater percentage of positives more quickly than either of the other 2 methods. Of the various media used for the isolation of the tubercle bacilli, Corper, Hohn, and Miraglia were found to be suitable for routine diagnostic cultures. The Hohn medium is made by the addition of a small amount of methyl green to sterile liquid egg white. The Corper medium is made according to his standard method (see Bacteriology). The Petrik medium is excellent and is highly recommended. More recent media are Tarshis and Whalen.

Corper's standard method of cultivation of tubercle bacilli: Prepare Corper glycerol water crystal violet paper medium by cutting large clean potatoes into cylinders that are soaked 1-2 hr. in a 1% sodium carbonate solution, which contains crystal violet in concentration of 1:75,000. This dye must be added just before use since the mixture tends to decolorize. These potato cylinders are wiped clean and placed in sterile tubes containing 1.5 ml. 6% glycerol water and sterilized at 120° C. 15 lbs. pressure for 30 min.

Malignant tumors of kidneys

In malignant tumors of the kidneys, we often find hematuria, intermittent or constant, as a urinary sign. In about ¾ of the cases it is the first definite symptom.

Renal calculus

In stone of the kidney the urine is somewhat concentrated, with high color and strongly acid reaction. Small amounts of albumin and a few casts may be present as a result of kidney irritation. The finding of blood cells is a very important suggestive fact in this disease, especially during the day and after severe exercise. The presence of a stone usually produces pyelitis, and variable amounts of pus then appear, the urine remaining acid in reaction. Diagnosis is confirmed by catheterization, x-ray, etc.

Pyelitis

In pyelitis the urine is slightly acid and contains a small or moderate amount of pus, together with many spindle and caudate epithelial cells. These findings may be intermittent, because of occasional blocking of the ureter on the diseased side. Pus casts and also other froms may appear when the process extends up into the kidney tubules. Albumin is always present. Even when pus is scanty, albumin is rarely under 0.15%. In all these cases, of course, bacteriologic studies must be made. *Proteus vulgaris* is found in about 50% of the cases.

Cystitis

In acute and in many chronic cases of moderate severity the urine is acid and contains a variable amount of pus, with many epithelial cells from the bladder, which are large, round, and pyriform cells. Albumin is present in small amount—less than 0.15%. *Escherichia coli* is most frequently found in these cases. In old chronic cases, especially when due to prostatic obstruction, the urine is alkaline. The sediment contains amorphous phosphates and crystals of triple phosphate and ammonium biurate. Many organisms are present.

Stone in bladder, tumors, and tuberculosis

These conditions will produce chronic cystitis, with its characteristic urine. Blood is more frequently present and is more abundant than in ordinary cystitis. With neoplasms, especially, considerable hemorrhage is likely to be found, and sometimes particles of the tumor are passed with the urine. In cases of tuberculosis it is comparatively easy to find tubercle bacilli.

Diabetes insipidus

Diabetes insipidus is characterized by an excretion of increased quantity of pale watery urine. The specific gravity is very low, around 1.001. The urine shows no sugar. There is, however, a marked increase in urea in many cases of diabetes insipidus.

Diabetes mellitus

Tests for diabetes mellitus are given in the discussion on clinical chemistry, pp. 89ff. The quantity of urine is increased up to as high as 4 L. in 24 hr. The urine is pale. The specific gravity is high, around 1.030-1.040. The urine shows the presence of glucose to a greater or lesser extent. Blood sugar is above normal. The quantity of glucose in the urine varies from 2 to as much as 8%. In advanced cases acetone is present; in very severe cases diacetic acid and oxybutyric acid appear.

Renal diabetes

In this disease there is no increase in blood sugar. There is a condition of lowering of the threshold point; that is, instead of no sugar appearing in the urine until the blood sugar reaches 170 mg.% or more, sugar appears in the urine with perfectly normal blood sugar content. It seems to be a disease of the filtration process of the kidney or represents a disturbance of the permeability of the kidney for sugar.

Nephrosis and nephritis

The meaning of the term **nephritis** in the light of present knowledge of pathology of the kidney has been changed to mean only those conditions that are **inflammatory** in nature. The primarily **degenerative** forms of kidney disease are called **nephroses.** The arteriosclerotic kidney, which is a result of changes in the kidney due to arteriosclerosis of the renal arteries, is not classified as nephrosis. Amyloid disease, which starts as a degenerative change in the vessels, is a nephrosis.

Nephroses are those diseases characterized anatomically by primarily degenerative lesions of the renal parenchyma. According to Fishberg,[70] nephroses may be divided into 4 groups as follows:

1. Larval nephroses, in which the renal lesions are of comparatively slight degree and are manifested clinically by albuminuria and the presence of some formed elements in the urine in the absence of impairment of renal function.
2. Necrotizing nephroses, in which there is necrosis of the tubular epithelium. In these cases renal insufficiency is common.
3. Chronic nephroses, in which there is chronic degenerative process in the renal epithelia, with edema as the most striking clinical feature.
4. Amyloid nephrosis.

Among the larval nephroses may be catalogued febrile albuminuria, diabetic nephrosis, nephrosis due to long-standing jaundice, pernicious anemia, and Graves' disease.

Of the necrotizing nephroses we are concerned with that type seen in acute mercury poisoning.

Chronic nephrosis is characterized clinically by edema and severe albuminuria with no arterial hypertension. Anatomically in these cases are found degenerative lesions of the renal parenchyma. Chronic nephrosis is the present term for what was formerly called parenchymatous nephritis, the large white kidney of the pathologic anatomist.

The etiology of chronic nephrosis is as follows: It is a disease of childhood and young adult life, decreasing in frequency with advancing years. Infection is supposed to play an important role in this condition. Of all the infectious agents the *Treponema pallidum* is the most clearly defined. Diphtheria, as well as pneumococcal infections, has been known to have been complicated by chronic nephrosis.

In chronic nephrosis one may find edema, proteinuria, diminution in the colloid osmotic pressure, lipemia, no impairment of renal function, arterial hypertension, and no primary inflammatory lesion of the kidney.

Albuminuria is the most common feature of chronic nephrosis. Slight and temporary glycosuria is sometimes found. In the sediment doubly refractive lipoids are found, first described by Munk. In order to determine the presence of these bodies one must use a polarizing device that can be attached to any microscope. In the blood one finds a decrease in the total protein content of the plasma, an increase in the fibrinogen content of the plasma, a decrease in the colloid osmotic pressure of the plasma, an increase in the velocity of sedimentation of the red blood cells, and an increase in the concentration of fats and lipoids in the plasma.

Amyloidosis: In amyloid nephrosis the amount of protein in the urine is very great; as much as 30 gm. protein may be lost in the urine daily. The blood picture in these cases shows profound anemia. There is sometimes a hypercholesterolemia. Edema is a common clinical symptom.

Nephritis: The type of nephritis that is most common is the acute glomerulonephritis secondary to infection, such as scarlet fever, tonsillitis, subacute bacterial endocarditis and pneumonia.

The urine is diminished, blood is present, and albuminuria is present in all cases, usually 0.2-0.4%. In the sediment, in addition to red cells and urates, we find casts—hyaline and blood casts, sometimes fatty and rarely waxy casts—leukocytes in great numbers, and epithelial cells. Specific gravity of the urine is high. There is usually impairment of renal function with retention of the nonprotein nitrogenous constituents. Nonprotein nitrogen of the blood is usually not markedly elevated, but occasionally may be as high as 180 mg./100 ml. In some cases concentration of serum chloride is high, in others low. Total protein content of the plasma is within normal limits.

See p. 1899 for further discussion of this condition.

Chronic glomerulonephritis: This is a condition of an unresolved acute glomerulonephritis. There are 3 types of chronic glomerulonephritis: the nephrotic type with edema, the hypertensive type, and the recurrent type. The amount of urine is decreased, and the specific gravity is low. The nonprotein nitrogenous constituents in the blood show no changes so long as renal function is unimpaired. With the beginning of renal decompensation retention occurs. Albuminuria is constantly present. Casts of all varieties are found in the urine—hyaline, granular, epithelial, and fatty.

Focal nephritis: According to Fishberg the term focal nephritis is used to indicate a nephritis that is a manifestation of infection, occurring at the height of the infection. Fishberg emphasizes, as Volhard[71] has already pointed out, an important difference between focal nephritis and acute glomerulonephritis, "for the latter most often, though not invariably, occurs after the primary infection has started to subside, as is seen in glomerulonephritis complicating scarlet fever and often tonsillitis. The close linkage of focal nephritis to the height of the infection is well illustrated by the old observation of Kannenberg[72] that in focal nephritis complicating relapsing fever the renal process waxes and wanes with the periods of the disease."* It is probable that the lesion in focal nephritis is due to the direct action of microorganisms on the renal structures. The types of infection in which this condition occurs are as follows: streptococcal infections, pneumonia, typhoid fever, and rarely in scarlet fever, measles, cerebrospinal fever, influenza, rheumatic fever, and many others.

In these cases hematuria is the leading symptom, occurring at the height of the infectious process. Together with blood in the urine, we find albumin, moderate in quantity, and varying numbers of hyaline, granular, and blood casts. Leukocytes are abundant, and epithelial cells may be present. In cases complicating tonsillitis we are likely to find bacteria in the stained sediment. Renal function is usually not impaired to any great degree, although the volume of urine is diminished.

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*From Fishberg, A. M.: Hypertension and nephritis, Philadelphia, 1934, Lea & Febiger.

Nephropathies due to vascular disease: In the majority of elderly persons there is some sclerosis of the renal arteries which extends into the prearterioles. This form of renal disease is called the arteriosclerotic kidney, and it may be found in persons who have had no signs of renal disease or hypertension.

Arteriolosclerotic or hypertensive kidney: In this condition there is disease of the small arteries and afferent glomerular arterioles, with persistent elevation of the blood pressure. The patient sooner or later develops signs of involvement of the heart, brain, or kidneys. In these cases death occurs from congestive heart failure, coronary disease, cerebral vascular accidents, or renal insufficiency. Autopsies on many cases of primary hypertension have shown normal-sized kidneys with finely granular surfaces. The so-called "primary contracted kidney" is a hypertensive condition with a high degree of atrophy. One must distinguish chronic glomerulonephritis from primary hypertension with uremia since the 2 diseases are different in etiology.

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Section Two

GASTRIC SECRETION AND DUODENAL CONTENT

Examination of the contents of the upper gastrointestinal tract contributes information pertinent not only to the function of the stomach and the duodenum but also of the accessory organs of the alimentary canal such as the gallbladder and the pancreas. As a result of the many changes in concept regarding diseases of the gastrointestinal tract, however, the analysis of gastric and duodenal contents has not proved as valuable as originally anticipated.

The most frequent assay concerned with gastric analysis is the determination of the presence or absence of free acid, both before and after stimulation. In this regard, however, a choice must be made as to the method of obtaining a suitable specimen. When properly performed, intubation of the patient is usually an innocuous procedure, although it does involve some discomfort. The tubeless test now available appears to be quite satisfactory and has an obvious advantage over the classical method.

Examination of the duodenal contents is at the present time used most often in conjunction with various research programs. The relative difficulty in obtaining the specimen and the lack of clinical applicability of the data obtained have resulted in little use of the available tests in this area. Information concerning the structure or mechanical function of organs such as the stomach, small intestine, and gallbladder is better obtained by radiologic examination, and abnormality of pancreatic function may be detected by the measurement of the level of activity of various pancreatic enzymes found in serum or urine.

Chapter 105

Gastric secretion

STANLEY REITMAN
SAM FRANKEL

GENERAL CONSIDERATIONS

Gastric contents

The clinical laboratory is concerned frequently with the determination of the chemical analysis of so-called "gastric contents." Gastric contents in this sense means material that remains in the stomach 1 hr. after a classical test meal has been administered. The test meal that has been in use for many years is the so-called "Ewald breakfast," later to be described. The purpose of placing a sample meal in the stomach is to determine after 1 hr. just how gastric activity has affected the material during this period. Normal gastric digestion is at its height 1 hr. after the administration of the meal.

Reviewing the physiology of gastric digestion, it is known that the secretion of gastric juice begins soon after food is placed in the stomach. It is a transparent, light-colored, yellow fluid which is acid in reaction and contains about 1% of solids. The specific gravity of gastric juice is very low, varying from 1.001 to 1.010. The principal phenomenon that goes on in gastric digestion is the splitting of the protein constituents of food by pepsin in the presence of hydrochloric acid. The action of rennin on milk is also of importance. Pepsinogen and renninogen are secreted by the gastric glands and are converted into pepsin and rennin by the action of hydrochloric acid. Hydrochloric acid combines loosely with the proteins and is called "combined" hydrochloric acid. That part of hydrochloric acid that is formed after the proteins have been converted into acidmetaprotein is designated "free" hydrochloric acid.

At the time the gastric contents are removed, namely, 1 hr. after the meal has been taken, **normally** the contents consist of water, hydrochloric acid, combined hydrochloric acid, pepsin, rennin, mineral salts, acid phosphates, and the remnants of undigested food. It also contains various products of digestion in solution. In pathologic conditions these materials are present in greater or lesser amounts and sometimes some of them are absent. Under certain conditions lactic acid is present. Microscopically, structures such as blood cells, bacteria, and abnormal cells may be seen.

Facts about gastric secretion

The stomach normally secretes hydrochloric acid, pepsin, rennin, and mucin. It is important for the laboratory to assist in estimating the degree and activity of the secretory function. Gastric acidity is an index to the concentration of the protein-splitting enzyme pepsin and the milk-clotting enzyme rennin. There are 2 kinds of mucin present in gastric secretion, a soluble mucin which plays an important part in buffering the acid and, in addition, the glairy mucus that is not concerned with this function.

According to Meakins[1] the amount of increased gastric secretion is best determined in a fasting state, and at this time the normal stomach should contain less than 100 ml. of more or less opaque liquid which, although acid to phenolphthalein, should not contain more than a low (under 10%) concentration of free hydrochloric acid. Greater quantities than 100 ml., and particularly if the amount of free hydrochloric acid is high, are significant of hypersecretion. Decreased secretion of acid is of considerable diagnostic importance in organic gastric lesions and systemic diseases, and such a decrease even to absence of hydrochloric acid is a common accompaniment of carcinoma of the stomach. However, since it occurs in 10-15% of normal people, it cannot be taken too seriously unless substantiated by other evidence of local disturbance. Meakins called attention, further, to lowered hydrochloric acid, or its absence as a prominent sign in the diagnosis of certain diseases of the blood, particularly pernicious anemia. The presence of free hydrochloric acid practically eliminates the diagnosis of pernicious anemia.

Meakins stated that gastric digestion depends almost entirely upon the presence of pepsin which is active in acid medium, but the determination of peptic activity is laborious and is seldom indicated. He believes that in rare cases in which there is an entire absence of acid secretion, determinations of peptic activity may be advisable.

METHOD OF OBTAINING STOMACH CONTENTS

Several kinds of tubes may be used in this technic, such as the Rehfuss, Ewald, or Levin tube. The Rehfuss tube consists of a small, metal oval tip attached to a very small caliber rubber tube. The tip is designed both for obtaining stomach contents and duodenal contents; i.e., it will slip through the pylorus into the duodenum after a certain period of time with the patient in correct position, lying on the right side.

The tubes are of course cleaned thoroughly and then placed in a basin of ice water before use. The patient should be gowned and in a sitting position, and the neck should be free. Gastric intubation should be performed only by a physician or a specially trained individual.

The cooperation of the patient is very important. He should be told just what is expected of him and what is about to transpire. He should be assured of the lack of pain and discomfort, provided he cooperates. He should be told to "swallow" the tube as soon as he is requested to do so. With the mouth wide open* and the head back a trifle, the head fixed in a steady position by the left hand of the operator, the right hand of the operator, without placing any fingers in the mouth, inserts the point of the tube directly in the middle line over the base of the tongue. The operator rapidly passes the tube into the posterior part of the mouth, almost "throwing" it in. The patient is now directed to swallow. At the same time the tube is pushed along gently and firmly with each swallow. Care must be taken that the tube, when placed over the base of the tongue, does not go into the trachea. The tube should not be oiled or lubricated since this material often causes the patient to vomit. Some individuals have a very active gag reflex, and the slightest pharyngeal stimulation may cause vomiting Between swallows the patient may breath shallowly and rapidly through his mouth (pant), in order to reduce the tendency to vomit. Provided there is no obstruction, the tube will glide rapidly into the stomach.

As soon as the tube is in the stomach, as judged by the mark on the tube, the outside end is lowered so that it will be below the level of the end in the stomach. A siphon is thus created and the material will flow out. If there is any difficulty, a clean glass 20 ml. syringe is attached to the outside end and suction is used. It is sometimes necessary to pull the tube gently in and out so as to remove any blocked food particles which may interfere with emptying the contents. If, in any way, failure occurs in inserting the tube, the patient must be assured that this sometimes happens and is not unusual, and that it is sometimes necessary to try again. The psychic influence of the patient in this procedure is very important. There should be no undue movement on the part of the operator or any signs of concern so that the patient's psyche will in no way be disturbed. The distance from the teeth to the cardiac orifice of the stomach is about 40 cm.

If the operator has any idea at the beginning of the procedure that the tube has gone into the larynx instead of into the esophagus, this idea may be dispelled or confirmed by holding the end of the tube to the ear and listening. If the intake and exhalation of air can be heard, the tube must be withdrawn. The tube does not go into the trachea ordinarily because the opening quickly closes at the approach of a foreign body. If there is considerable disturbance and severe dysphagia and continued strong convulsive coughing during the beginning of the placing of the tube, this indicates that the tube is in the fossa pyriformes at the side of the larynx and the tube is probably rolled up in a spiral. It must be withdrawn and started again. If it is not taken out immediately, it will suddenly be regurgitated after it is drawn up into the mouth.

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*When a Levin type tube is used, the physician may prefer to pass it through the nose.

After the stomach contents have been removed, the tube should be pinched securely with the fingers and rapidly withdrawn. This pinching of the tube holds any foreign body that may be in the tube securely during the process of withdrawal and thus prevents the dropping of foreign bodies into the posterior pharynx and possible entrance into the trachea.

TUBELESS GASTRIC ANALYSIS (DIAGNEX BLUE)*

Azure A is exchanged from an Amberlite cation resin by hydrogen ions from hydrochloric acid of the gastric juice following caffeine stimulation. The azure A is rapidly absorbed from the small intestine and excreted in the urine. The approximate concentration may be estimated by the intensity of the color when compared with permanent standards supplied with the test material.

Directions to patient: The patient must be in the postabsorptive state until the test is completed.

1. Discard the urine specimen voided upon arising.
2. Take the 2 tablets contained in the small package with 1 glass of water.
3. At the end of 1 hr. void and discard the specimen.
4. Pour the granules from the large package into ¼ glass of water, stir well, and drink. The granules do not dissolve and should not be chewed. Rinse the glass with a little more water for any remaining granules.
5. At the end of 2 hr. collect the complete urine specimen in a clean jar.

The patient may eat at this time.

Procedure:

1. Dilute the urine specimen with water to 300 ml.
2. Fill 3 test tubes with approximately 10 ml. of the specimen.
3. Add approximately 300 mg. ascorbic acid to 2 of the test tubes. This will remove any color so that they may be used as controls by placing them in the comparator slots supplied with the permanent standards. Place the untreated sample in the center slot provided for the test specimen.

If the color intensity of the test urine is equal to or exceeds that of the 0.6 mg. standard, the patient has secreted free gastric hydrochloric acid and the test is complete.

If the test aliquot is less intense in color than the 0.6 mg. standard, acidify with 1 drop of Diagnex blue reagent (or 2 drops of 10% hydrochloric acid). Heat all 3 tubes in a boiling water bath for 10 min. Remove the tubes and allow them to cool for 2 hr. Compare color intensity as before.

If the color of the test specimen falls between the 0.6 mg. and the 0.3 mg. standards, it is presumptive evidence of hypochlorhydria.

If the color of the test specimen is less intense than that of the 0.3 mg. standard, it is presumptive evidence of achlorhydria.

Comments: The color comparator supplied with the test materials has standards that represent 0.3 and 0.6 mg. azure A.

Since azure A may be excreted in the urine in either the blue form or a conjugated colorless form, false positive tests (lack of color) may be avoided by heating in acid solution which oxidizes the conjugate to the colored form.

The blue color, which may disappear on boiling, reappears upon cooling. The full 2 hr. should be allowed for complete color development.

The use of ascorbic acid, which reduces azure A to the colorless form, allows this test to be done with only a single specimen.

Blue or green urine which may be passed for a few days following the test has no significance.

Segal et al.[2] have adapted this test to serum which avoids the difficulties encountered in cases of renal insufficiency or when it is suspected than an incomplete specimen has been submitted.

TEST MEAL EXAMINATION

The usual method of examination of the stomach contents is one examination obtained 1 hr. after a test meal.

Test meals are given to stimulate secretion of the stomach with food. It can be stimulated with various foods, but for the sake of obtaining uniform results certain standard test meals have been adopted.

Test meals are given in the morning because the stomach is usually empty at this time. If there is reason to believe the stomach will not be empty in the morning, it is well to wash it with water the evening before.

Ewald test breakfast: This consists of 40 gm. bread (1 roll or 2 slices of bread) and 400 ml. water or weak tea without cream or sugar. It should be well masticated.

Dock test breakfast: Same as the Ewald breakfast except that a shredded whole wheat biscuit is substituted for the bread. This substitution is carried out because the Ewald test meal introduces with the bread a variable amount of lactic acid and numerous yeast cells, which are completely eliminated by the shredded wheat meal. The shredded wheat biscuit contains no lactic acid.

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*E. R. Squibb & Sons, New York, N. Y.

Boas test breakfast: This consists of a tablespoonful of rolled oats in a quart of water, boiled to 1 pint, with a pinch of salt added. This should be withdrawn in 45 min.-1 hr. after introduction. It does not contain lactic acid. When this meal is used, the stomach should always be washed with water the evening before.

Riegel test meal: This consists of 400 ml. bouillon, a broiled beefsteak (about 150-200 gm.), and 150 gm. mashed potato. It must be thoroughly masticated; otherwise the tube may be occluded when attempt is made to withdraw the meal.

Fischer test meal: This is similar to the Riegel, consisting of an Ewald breakfast plus ¼ lb. lean, finely chopped Hamburg steak, broiled and lightly seasoned. The Fischer meal and the Riegel meal may be removed in 3-4 hr. They give higher acidity values than the Ewald breakfast.

Histamine test: This is a test to demonstrate the ability of the stomach to secrete free hydrochloric acid under the maximum amount of stimulation. It should be done as an initial test if pernicious anemia is suspected. It should also be done as a control on any patient showing achlorhydria or hypochlorhydria with the Ewald test meal. In other words, if the gastric analysis shows an achlorhydria or hypochlorhydria after using this powerful stimulating agent, the results are much more significant than the lowered value for hydrochloric acid or the absence of hydrochloric acid after an Ewald meal. **This is administered only on order of a physician.**

The test should be performed with the patient under as nearly a basic condition as possible. Remove the stomach contents of a fasting patient by means of a gastric tube. Then administer histamine hydrochloride or histamine phosphate hypodermically. Gompertz and Cohen[3] recommend a total dose of 0.25 mg. This can be most advantageously administered by injecting 0.25 ml. of a sterile 1:1000 solution of histamine subcutaneously. Completely aspirate the stomach contents at 30 min. intervals from the time of injection and determine the volume and total acidity of the samples.

Alcohol test of gastric function: The use of alcohol with phenolphthalein added as an indicator has been suggested as a substitute for test meals. The technic is to have the patient swallow 49 ml. 7% solution of alcohol to which has been added 1 ml. 0.1% phenolphthalein in 90% alcohol.

PRELIMINARY SURVEY

Volume: Normal stomach yields about 20-50 ml.

Color: Normal is colorless. It may be green from bile. Blood is characteristic when present. Color may be altered by blood or drugs.

Consistency: Normally it is clear. It may contain mucus or undigested food particles.

Odor: Normally odorless. In disease the odor is sour or rancid. Watch for the odor of drugs.

Sediment: Normally none. May contain pieces of mucus, food remnants, blood clots, tumor particles.

Starch: Add 1 drop Gram's iodine to the gastric contents. Should starch be present, it will turn blue if undigested. If partially digested, it will turn red.

Reaction: The reaction of gastric contents is normally acid. It may be neutral in some cases of gastric cancer and marked chronic gastritis or when contaminated by a considerable amount of saliva.

Mucus: A small amount of mucus is normally present, but large amounts suggest chronic gastritis.

Bile: Bile is seen in very minute particles as the result of excessive straining while the tube is in the stomach. Large amounts are rarely found but if they are, it indicates upper intestinal obstruction.

CHEMICAL EXAMINATION OF GASTRIC CONTENTS

Routine examination means a qualitative test for free hydrochloric acid and organic acid and quantitative estimations of total acidity and free hydrochloric acid. In routine work qualitative tests are performed before quantitative tests are begun.

Topfer test for free hydrochloric acid

Qualitative

Reagent:

1. Topfer reagent:

Dimethylaminoazobenzol	0.5 gm.
Alcohol, 95%	100 ml.

Procedure:

1. Place a few drops of gastric juice in a test tube.
2. Add 1 drop Topfer reagent.

A bright red color will appear if free hydrochloric acid is present.

Quantitative

Reagents:

1. Topfer reagent (above).
2. N/10 Sodium hydroxide.
    (a) To about 50 ml. distilled water in a 100 ml. volumetric flask add 10 ml. N/1 sodium hydroxide. Dilute to 100 ml. with distilled water.

Procedure:

1. Place 10 ml. gastric juice in a casserole. Add 1 drop Topfer reagent. A bright red color develops if free hydrochloric acid is present.
2. Place N/10 sodium hydroxide in a buret. Add the sodium hydroxide from the buret drop by

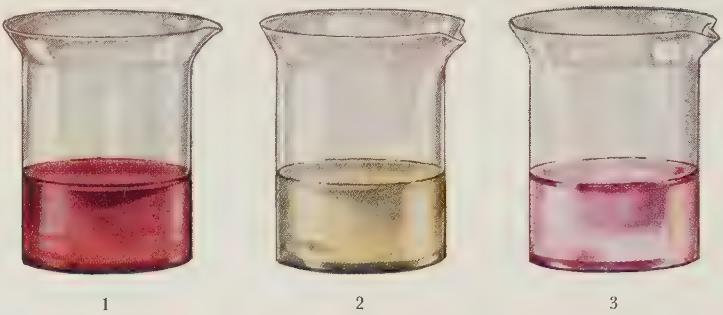

Fig. 680. Titration of stomach contents. **1,** Contents with Topfer's reagent, showing presence of free hydrochloric acid. **2,** End point in titration for free hydrochloric acid. **3,** End point in titration for total acidity in the presence of phenolphthalein.

drop to the solution in the casserole until the red color is replaced by a canary yellow. Stir constantly during the addition of the sodium hydroxide.

3. Read from the buret the number of ml. sodium hydroxide used. Calculate the number of ml. sodium hydroxide required for 100 ml. gastric juice. This gives the degree of acidity.

Example: 2 ml. N/10 sodium hydroxide were used for 10 ml. gastric juice. Ten times 2 ml., or 20 ml., would be required for 100 ml. gastric juice. The degree of free hydrochloric acid is therefore 20.

Normal free hydrochloric acid is 20°-40°.

To change the degree of acidity to percentage, multiply by 0.00365.

Degrees are numerically equal to mEq./L.

It is sometimes advantageous to determine the amount of hydrochloric acid deficiency in cases of achlorhydria. To do this test place 10 ml. gastric contents in a casserole, add 2 drops Topfer reagent, and titrate with N/10 hydrochloric acid until the first red indicative of free hydrochloric acid appears. Calculate the amount of N/10 hydrochloric acid required for 100 ml. gastric contents; this figure represents the degree of hydrochloric acid deficiency. This is not a routine test and is useful only in those cases in which hydrochloric acid therapy can be used to correct the hydrochloric acid deficiency.

Total acidity

The total acidity comprises free hydrochloric acid, loosely combined hydrochloric acid, acid salts, and organic acids such as lactic acid, butyric acid, and amino acids.

Reagents:

1. Phenolphthalein, 1% alcoholic.

Phenolphthalein	1 gm.
Alcohol, 95%	100 ml.

2. N/10 Sodium hydroxide.

Procedure:

1. Place 5 ml. unfiltered gastric contents in a casserole. Add 2 drops 1% alcoholic phenolphthalein.
2. Have a buret filled with N/10 sodium hydroxide. Add the sodium hydroxide from the buret a drop at a time to the mixture in the casserole, stirring constantly, until the mixture remains a deep pink color. This is the end point. Read from the buret the number of ml. sodium hydroxide used. Calculate the number of ml. sodium hydroxide required for 100 ml. gastric contents. This gives the degree of total acidity.

Example: If 3 ml. N/10 sodium hydroxide were used to neutralize 5 ml. gastric contents, multiply 3 ml. by 20 = 60 ml., or the degree of total acidity in 100 ml. gastric contents. **Normal** degree of total acidity is between 40 and 60-70.

Lactic acid

Lactic acid is not found normally in gastric juice. It may be present after ingesting certain foods, e.g., sour milk or foods prepared with it. If emptying of the stomach is delayed or if the secretion of hydrochloric acid is low, lactic acid may be formed from fermentation of carbohydrates by such lactic acid–forming organisms as the Boas-Oppler bacillus. This is a common finding in advanced carcinoma of the stomach.

Reagents:

1. Phenol, 1%.

Liquefied phenol	1 ml.
Distilled water to make . .	100 ml.

2. Ferric chloride, 10%.

Ferric chloride	10 gm.
Distilled water to make . .	100 ml.

Procedure:

1. To 15 ml. 1% solution of phenol in a test tube add 10% ferric chloride until an amethyst color is produced. Usually 1 or 2 drops are enough.
2. Add a few drops of unfiltered gastric contents.

A canary yellow color indicates lactic acid.

Kelling method

1. Fill a test tube with distilled water.
2. Add 2 drops 10% ferric chloride. Mix.
3. Divide the contents of the test tube into 2 parts.

4. In the first half place 1 ml. filtered gastric juice.
5. If lactic acid is present, a deep yellow color develops. Compare this color with the color of the control tube (distilled water plus ferric chloride).

Strauss method

1. Place 5 ml. filtered gastric juice in a small separatory funnel.
2. Add 20 ml. ether. Hold the stopper in tightly and shake thoroughly.
3. Allow to stand in an upright position until the ether separates.
4. Allow the bottom fluid to run out. Retain the upper 5 ml. of ether.
5. Add to this ether extract 20 ml. distilled water and 2 drops 10% ferric chloride solution.
6. Shake gently.

A slight green color indicates the presence of 0.05% lactic acid. An intense yellowish green color indicates the presence of 0.1% lactic acid.

Uffelmann method

Reagent:

1. Add 3 drops liquid phenol to 3 drops 10% ferric chloride. Dilute with distilled water until an amethyst color is produced.

Procedure:

1. To 50 ml. ether add 5 ml. filtered gastric juice. Shake thoroughly for 10 min.
2. Collect the ether by means of a separatory funnel or a dropper pipet.
3. Evaporate to dryness. Add to the residue 5 ml. distilled water.
4. Add the dissolved residue to Uffelmann reagent.

A canary yellow color indicates the presence of lactic acid.

Achlorhydria

The continued absence of free hydrochloric acid in the stomach contents is not in itself a disease entity. There are very few diseases characterized by achlorhydria. In these cases additional clinical and laboratory findings will aid in the proper diagnosis. The lack of titratable acidity in some patients is due to bile and duodenal regurgitation which neutralizes the acid output of the stomach. This kind of achlorhydria can be determined by the estimation of chlorides and trypsin in the gastric contents. Although no free acid may appear in the first hour, the study of an acid curve which appears after 60 min. may be helpful. Achlorhydria is found in the following diseases: pernicious anemia, diabetes mellitus, chronic gastritis, carcinoma of stomach, pellagra, syphilis, amebic dysentery, achylia gastrica, hypothyroidism, rheumatoid arthritis, and pulmonary tuberculosis.

Pernicious anemia: There is very seldom any free hydrochloric acid present in pernicious anemia. The enzymes rennin and pepsin are also absent. The absence of free hydrochloric acid and of the enzymes in the gastric contents may be found long before the blood picture becomes characteristic. Even though liver therapy or vitamin B_{12} may bring about a normal blood count, the achlorhydria remains.

Diabetes mellitus: In certain cases of this disease with lack of appetite there is absence of or great decrease in free hydrochloric acid. The symptoms improve when the patients are given dilute hydrochloric acid.

Chronic gastritis: In this condition the acidity may vary from time to time. Gastritis of the fundus tends toward the absence of hydrochloric acid.

Carcinoma of stomach: Absence of free hydrochloric acid, lactic acid in the gastric contents, occult blood in the stool, defect and slow emptying of the stomach as noted in x-ray examination, all favor the diagnosis of malignancy.

Pellagra: Achlorhydria is often encountered in this disease.

Syphilis: There is sometimes present a gastric subacidity, anacidity, or complete achlorhydria in this disease.

Amebic dysentery: Porgess states that in amebic dysentery the gastric contents frequently show achlorhydria.

Achylia gastrica: Here free hydrochloric acid is absent, and the total acidity rarely rises above 10. The gastric enzymes are absent, due to atrophy of the mucosa. No gastric secretion follows the injection of histamine. Normally 0.1 mg. histamine/10 kg. body weight, injected hypodermically, will stimulate gastric secretion.

Miscellaneous conditions: Hypothyroidism, rheumatoid arthritis, and pulmonary tuberculosis may frequently be associated with achlorhydria.

Hyperacidity

Hyperacidity is a common occurrence. It is found in patients with neuroses, in cases of constipation, and in excessive cigarette smokers. The eating of too many pickles, of foods rich in condiments, or of excessive amounts of carbohydrates may produce it.

Gastric and duodenal ulcers are frequently found in association with high acidity; however, in about 50% of gastric ulcer cases the acidity is normal. In duodenal ulcer the high acidity factor is more constant. The point of high acidity is usually reached in 45 min.-2 hr. after eating the test meal.

Occult blood

Benzidine test

1. Prepare about 3 ml. saturated solution of benzidine (special for blood) in glacial acetic acid in a test tube.
2. Add an equal volume of 3% hydrogen peroxide. Mix thoroughly. Should the solution turn green or blue, it must be discarded.
3. Add a few drops of unfiltered gastric contents and shake.

A green or blue color indicates the presence of occult blood (p. 1835).

Teichmann test for hemin crystals

Disintegrated blood must be examined chemically by the hemin test of Teichmann.

1. Mix a small amount of the suspected material with 1-2 crystals common salt, or place it on the thin layer of salt formed by slowly evaporating a small drop of physiologic salt solution on a slide.
2. Cover with a cover glass and run in enough glacial acetic acid to fill up the space between slide and cover.
3. Warm the slide over a flame for ¾-1 min. until bubbles arise, adding more glacial acetic acid until a faint reddish brown tint appears.
4. Then let the acetic acid evaporate entirely, and run distilled water in from the edge of the cover glass.

Microscopic examination will show dark brown rhombic plates or columns of hemin if blood is present.

Benzidine dihydrochloride test

Follow the method given on p. 1835, substituting gastric contents for urine.

Benzidine test—method of Levin-Watt[4]

1. Mix the gastric or duodenal contents thoroughly with an equal volume of distilled water.
2. Filter through 1 or 2 Whatman No. 5 extraretentive filter papers to obtain a clear filtrate.
3. To 3 ml. clear filtrate add 5 drops 50% acetic acid and 5 drops hydrogen peroxide and shake vogorously.
4. Overlay the mixture with 0.5 ml. 6% alcoholic benzidine solution and rock slightly. The benzidine solution should preferably be 2 or 3 days old.

A positive reaction is indicated by a green ring at the junction of the benzidine solution and the filtrate mixture.

This test can be used for detecting **blood in vaginal washings.** Sterile water irrigation of the posterior portion of the vagina is performed with an Asepto syringe of 10-20 ml. capacity.

Perform the test without filtration, and, if positive, follow by Papanicolaou examination. The test is positive in cases in which there are 4-6 red blood cells/high-power field.

Occult blood in urine:

1. If urine is to be tested, centrifuge 10 ml. at 1500 r.p.m. for 3 min., decant the supernatant fluid, and use the sediment.
2. Add 1 ml. water and 3 drops 50% acetic acid, shake, and add 3 drops of hydrogen peroxide. Shake vigorously.
3. Overlay the mixture with 0.5 ml. 6% alcoholic benzidine solution and observe for a green ring at the point of contact of the liquids.

Occult blood in feces:

1. If feces are used, emulsify a small portion in distilled water, filter as for gastric contents, and use 3 ml. filtrate, 8 drops acetic acid, and 8 drops of hydrogen peroxide before overlaying with the alcoholic benzidine solution.

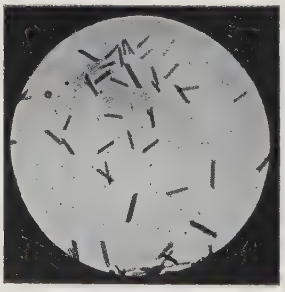

Fig. 681. Hemin crystals. (×100.)

Bile

Break up a match stick in about 5 ml. nitric acid (concentrated). Stratify with a few ml. gastric juice.

A play of colors will develop at the point of contact if bile is present.

Butyric acid

Butyric acid is manifested by the rancid, butterlike odor. If a test is desired, boil the gastric contents to accentuate this odor.

If a piece of moistened blue litmus paper is held in the mouth of the tube, the volatile acid reddens it as it escapes during the boiling. Butyric acid also separates as a drop of oil if a piece of calcium chloride is added to it.

Rennin-renninogen

Rennin is the milk-curdling enzyme of the gastric juice. It is derived from renninogen through the action of hydrochloric acid. Deficiency of rennin has the same significance as deficiency of pepsin and is more easily recognized.

Test

1. Neutralize 5 ml. filtered gastric juice with very dilute sodium hydroxide solution.
2. Add 5 ml. fresh milk and place in an incubator or in a vessel of water at about 40° C.

Coagulation of the milk in 10-15 min. shows a normal amount of rennin. Delayed coagulation denotes a less amount.

Pepsin*

Tests for identity

"An aqueous solution of pepsin (1 in 50) is acid to litmus paper. An aqueous solution of pepsin yields precipitates with solutions of tannic or gallic acid and with solution of the salts of many heavy metals.

"On heating a saturated solution of Pepsin in acidulated water to 100° C., it becomes milky or yields a light, flocculent precipitate, and loses all proteolytic power.

"**Technic:** Mix 25 c.c. of normal hydrochloric acid with 275 c.c. of distilled water. Dissolve 0.1 gm. of Pepsin in 150 c.c. of this acid. Select a hen's egg, which is not less than 5 and not more than 12 days old and has been kept in a cool place, and immerse it in boiling water during 15 minutes. As soon as the egg has sufficiently cooled to handle, remove the shell and pellicle and all of the yolk and at once rub the albumen through a clean, dry, hair or brass, No. 40 sieve, reject the first portion that passes through the sieve, and place 10 gm. of the succeeding portion in a wide-mouthed bottle of 100 c.c. capacity. Immediately add 20 c.c. of the acid and stir the mixture well with a rubber-tipped glass rod so as to separate thoroughly the particles of albumen from each other. Rinse the rod with 15 c.c. more of the acid and, after warming the mixture to 52° C., add exactly 5 c.c. of the acidulated solution of Pepsin. At once cork the bottle securely, invert it 3 times and place it in a bath that has been previously regulated to maintain a temperature of 52° C. Keep the bottle at this temperature for 2½ hours, agitating the contents every ten minutes by inverting it once. Then remove it from the bath, pour the contents into a conical measure having a diameter not exceeding 1 cm. at the bottom, and transfer the undigested egg albumen, which adheres to the sides of the bottle, to the measure with the aid of small portions (about 15 c.c. at a time) of distilled water, until the total amount used measures 50 c.c. Stir the mixture well and allow it to stand for half an hour. The deposit of undissolved albumen does not measure more than 1 c.c.

"The relative proteolytic power of Pepsin, stronger or weaker than that just described, may be determined by ascertaining through repeated trials the quantity of the Pepsin solution made as directed in the assay, required to digest, under the prescribed conditions, 10 gm. of boiled and disintegrated egg albumen. Divide 15,000 by this quantity expressed in c.c. to ascertain how many parts of egg albumen one part of Pepsin will digest.

"Preserve in well-closed containers."

MICROSCOPIC EXAMINATION

To make a microscopic examination place a drop of unfiltered stomach contents upon a slide, cover with a cover glass, and examine with a low dry and a high dry objective with the diaphragm opening much reduced. If a drop of diluted Lugol solution is placed on the edge of the cover glass, it will run under the cover by capillary attraction and will help to bring out certain structures.

Normally we see starch granules with an occasional epithelial cell, yeast cell, or bacterium. Starch stains blue with iodine solution when undigested, and reddish, due to erythrodextrin, when partially digested. These colors, however, are not shown clearly unless the iodine solution is very dilute.

Under pathologic conditions we see remnants of food from previous meals, red blood cells, pus cells, sarcinae, and excessive numbers of yeast cells and bacteria.

Remnants of food from previous meals indicate deficient gastric motility. Red blood cells are recognized by chemical tests, preferably the **benzidine** test. Microscopically the red blood cells retain a fairly normal appearance but are so degenerated at times that only granular pigment is left. The presence of very fresh-looking red cells is highly suggestive of irritation of the stomach mucosa by the stomach tube. Pus cells are rarely seen in gastric contents, although many of them have been seen in cases of gastric cancer. These cells are partially digested, only the nuclei remaining. The nuclei appear as highly refractile bodies.

Swallowed sputum may be present.

Sarcinae are small spheres arranged in cuboid groups, often compared to bales of cotton. They are easily recognized and stain brown with iodine solution. They are rarely found in cases of gastric carcinoma. A few yeast cells may be found under normal conditions with considerable numbers in retention and fermentation. They stain yellow to brown with iodine solution. The bacteria encountered are not very important except the Boas-Oppler bacillus. It is claimed that this is found in the majority of cases of carcinoma of the stomach and rarely in any other condition. This organism belongs to the *Lactobacillus bulgaricus* type. The Boas-Oppler bacillus is large, 5-10μ long, nonmotile, and arranged in clumps or in zigzag chains. It stains yellow to brown with iodine solution, thus distinguishing it from *Leptotrichia buccalis* which is sometimes swallowed and may be found in stomach fluid.

Crystalline elements in stomach lavage: Rafsky[5] reported that crystalline elements were present in the stomach lavage of patients with cholelithiasis. In an effort to corroborate this

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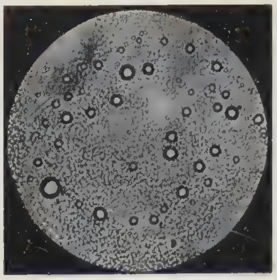

Fig. 682

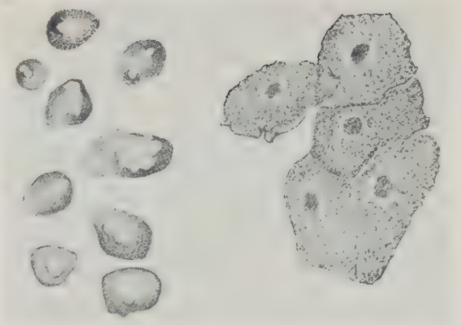

Fig. 683

Fig. 682. Fat globules. (×400.)

Fig. 683. Starch granules. Squamous epithelial cells.

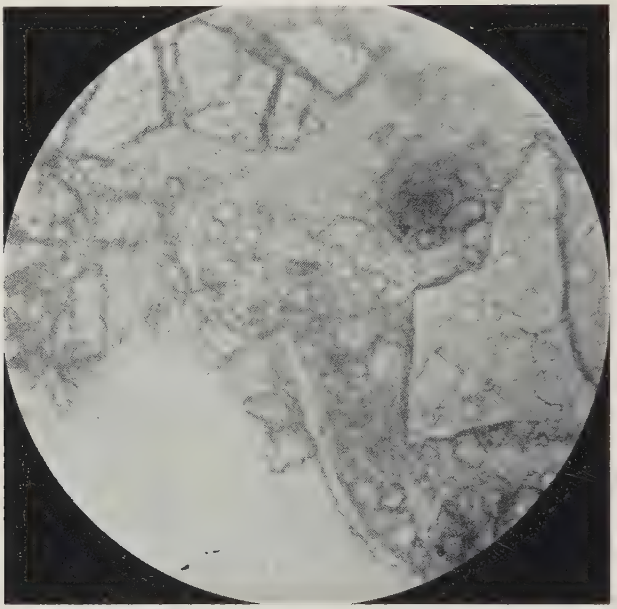

Fig. 684. Cholesterol and carbonate crystals; slight amount of calcium bilirubinate pigment. (Photomicrograph of particle in preoperative specimen of gastric lavage.) (From Rafsky, H. A.: J. Lab. & Clin. Med. **19**:959, 1934.)

finding, this subject was further investigated in a series of patients with and without biliary tract disease. This study was aided by the use of photomicrographs of crystals seen in the stomach lavage before operation, which were compared with photomicrographs of crystals found in the bile and stones removed from the gallbladder and bile ducts at the time of operation.

CONCLUSIONS TO BE DRAWN FROM THE EXAMINATION OF GASTRIC CONTENTS

A number of important points and diagnoses are deducible from examination of the gastric contents. First, the emptying time of the stomach is in a measure determined by this examination. The presence of blood in the gastric contents in visible amount is probably due to hemorrhage from an ulcer or carcinoma. Due consideration, of course, must be given to the possibility of a small amount of blood sometimes caused by the passage of the stomach tube.

If there is no free hydrochloric acid present after a test meal, one should consider the following conditions—pernicious anemia, primary hypochromic anemia, and cancer of the stomach. In pernicious anemia there is complete absence of hydrochloric acid, whereas in cancer a small amount may be present at times, although this is rare.

When increased hydrochloric acid is seen in patients with ulcers, it is due to hypersecretion by the parietal cells of the stomach. That there is a relationship between hydrochloric acid and peptic ulcer is evidenced by the fact that those with histamine achlorhydria usually do not develop either duodenal or benign gastric ulcers.

In **dilatation of the stomach** there is evidence of retention and fermentation, with hydrochloric acid diminished. Pepsin may be normal or slightly diminished. Lactic acid may be detected in small amounts, but is usually absent when the stomach has been washed before giving the test meal. Microscopically we find sarcinae and other bacteria and great numbers of yeast cells.

In **chronic gastritis** free hydrochloric acid may be increased in early cases, but it is usually diminished in well-marked cases and is often absent in advanced cases. Lactic acid may be present in traces. Pepsin and rennin are diminished in marked cases. The presence of large amounts of mucus is very characteristic of this condition.

In **atrophic gastritis** or **achylia gastrica** which is the terminal stage of chronic gastritis, associated with the blood picture of pernicious anemia there is decrease in with sometimes entire absence of free hydrochloric acid and proteolytic enzymatic activity. Total acidity may be down to 1°. Small amounts of lactic acid may be present.

Gastric carcinoma is possibly the most important condition which we have in mind in examination of gastric contents. There is a total absence of free hydrochloric acid and the presence of lactic acid and of the Boas-Oppler bacillus. Exceptions to this have been found where some free hydrochloric acid and no lactic acid were present in undoubted cases of carcinoma of the stomach. It must be borne in mind, however, that the presence of lactic acid is very strong presumptive evidence of the existence of carcinoma. In gastric carcinoma we often find blood, especially when the tumor is located at the pylorus. In these cases occult blood is also likely to be found in the stool.

Gastric ulcer is associated with an excess of free hydrochloric acid in about half of the cases. In other cases the acid is normal or diminished. The presence of blood is a very significant sign of ulcer. It is especially important in these conditions to make an examination of the stool for occult blood, taking care, of course, to have the patient on a meat-free diet for 3 or 4 days before making the test.

Summary: Examination of the gastric contents is of greatest value in the diagnosis of benign gastric ulcer, gastric cancer, and severe anemia of both pernicious and primary hypochromic type. In gastric ulcer, blood, an excess of hydrochloric acid, and perhaps gastric retention are found. In cancer there is very little or no hydrochloric acid; the presence of blood, together with gastric retention and lactic acid if the growth is near the pylorus, is characteristic. Finally, in pernicious anemia and primary hypochromic anemia there will be a complete absence of hydrochloric acid, no blood, no gastric retention or lactic acid.

GASTROSCOPY AS AN AID TO GASTRIC DIAGNOSIS

The following facts were well presented by Deutsch.[6] Gastroscopic study is indicated (1) in x-ray negative dyspepsia, (2) to clarify an indefinite roentgenographic impression or confirm a positive diagnosis of carcinoma, and (3) in all patients with gastric ulcer or polyps.

Gastroscopy can help in distinguishing benign from malignant ulceration of the stomach. In the antrum and middle portions of the stomach, observation of peristaltic waves and segmental tonus changes aids in ruling out an infiltrating rigid neoplastic lesion. Infiltrating lesions in the cardia or fundus show evidence of obstruction at gastroscopy as much as 6 mo. before it is detected by x-ray examination.

Positive biopsies have been taken during gastroscopy. Exfoliative cytology, accomplished by scraping with a polyethylene tube under gastroscopic observation, yields a desquamated mucosa and muscularis mucosa which is quickly aspirated, fixed in formalin, and prepared for cell block.

Attention is here called to the balloon technic of Cooper, described on p. 1781 on the exfoliative diagnosis of gastric lesions.

Radiology and gastroscopy are complimentary aids in the diagnosis of diseases of the stomach. It must be remembered that gastric secretion or mucosal folds in tetanic spasm in a very obese patient occur frequently and make mucosal relief studies by x-ray difficult. At gastroscopy these factors are no obstacle to an adequate examination.

PROTEIN NITROGEN AND NONPROTEIN NITROGEN DETERMINATIONS ON GASTRIC JUICE

Value: A series of papers has been written by Lay Martin[7, 8] on this question.

Normal nonprotein nitrogen is usually found within certain quantitative limits; the fasting juice in normal cases contains more protein and nonprotein nitrogen than the succeeding ones.

Benign achlorhydria, under stimulation with histamine, showed higher values than those of the normal but lower than those of gastric carcinoma or of nephritis with marked increase in blood nonprotein nitrogen.

Does the stomach act as an agent for removal of these products of metabolism from the body? Are they reabsorbed in the lower intestinal tract? Are they destroyed or benignly conjugated? Williams and Dick[9] attempted to answer these questions by establishing standards of normality for intestinal excretions of nonprotein nitrogen products after ingestion of magnesium sulfate. They showed they are quantitatively increased in nephritis.

Withdrawal of contents and precipitation of proteins: A duodenal tube is placed in the dependent portion of the stomach of a patient who has fasted for 15 hr., the position of the tube being verified by fluoroscopy. Entire fasting secretion is removed and placed in a refrigerator.

Gastric juice flow is stimulated by hypodermic injection of histamine (ergamine acid phosphate) in amounts equivalent to 0.005 mg./kg. body weight. Entire flow of gastric juice is collected by continuous siphonage into a narrow container surrounded by ice. All specimens with bile tinge are discarded. After gastric flow stops, all gastric juice is filtered in a refrigerator at 7° C., giving a limpid fluid free of mucus.

Proteins of the gastric juice are precipitated by tungstic acid at pH 3.5. A protein-free filtrate is obtained which can be used by methods described for the estimation of amino acid, urea, ammonia, and nonprotein nitrogen. Total nitrogen for the whole mixture is determined by the Kjeldahl method. The precipitate is redissolved in alkali and the protein nitrogen determined by the same technic.

Normal averages: Protein of the mucus-free gastric juice averaged 22.59 mg. **nitrogen**/100 ml. gastric juice.

The **nonprotein nitrogen** was 25.5 mg./100 ml. gastric juice.

Amino acid nitrogen averaged 7.22 mg. nitrogen/100 ml. gastric juice.

Urea averaged 2.58 mg. nitrogen/100 ml. gastric juice.

Ammonia nitrogen averaged 5.19 mg. nitrogen/100 ml. gastric juice, far more than the negligible amount in blood, suggesting speculation concerning the purpose of the urea-splitting enzyme appearing in the gastric juice and the gastric mucosa.

Uric acid averaged 1.5 mg./100 ml. gastric juice.

Accordingly, there are unaccounted 8 mg. nonprotein nitrogen.

Results in different cases: In benign achlorhydria, protein and nonprotein nitrogen double that of the control group. Compared with 7 cases of pernicious anemia, it was found that there was much more amino acid nitrogen and urea nitrogen in pernicious anemia than in benign achlorhydria with consequent rise in nonprotein nitrogen.

In proved cases of carcinoma of the stomach, protein and nonprotein nitrogen fractions increased from 4-6 times the normal.

Summary:

1. Normal gastric juice shows rather constant figures for protein and nonprotein nitrogen fractions (amino acid, urea, uric acid, and ammonia).
2. In benign achlorhydrias the amounts increased 2-fold.
3. In pernicious anemia the same increase was found plus still greater increase in amounts of amino acid and urea.
4. In carcinoma of the stomach with achlorhydria, very large amounts of protein and nonprotein nitrogen were found.

Methods for determination[8]

Total nitrogen has been determined for normal gastric juice by the Kjeldahl method.

To obtain a protein-free filtrate it was necessary to devise a method for the precipitation of these materials from a medium with a normal pH about 2; in the achylias it was about 7. Blood proteins are precipitated by tungstic acid according to the method of Folin and Wu at a pH of about 7. This method, when applied to gastric juice, in most cases gives no precipitate. Only when the hydrogen ion concentration has been increased to a pH above 4 is one able to precipitate the maximum amount of protein. Routinely this was done in the following manner.

Procedure:

1. To gastric juice add ½ the amount of 10% sodium tungstate. As this solution is gradually added, a cloudy precipitate is formed which is reabsorbed as the pH decreases.
2. Then add ⅔N sulfuric acid drop by drop until the pH of the solution is brought to about 3.5. From many trials it has been found that this is about the optimal point of the heaviest precipitation. This end point can be recognized by means of Congo red paper

which just begins to turn from light to dark blue.

3. Make the material to any required dilution, usually about 1:5. In the achylias, experience has shown that a 1:7 dilution is sufficient.
4. Filter through Whatman No. 42 filter paper which will usually produce a perfectly clear filtrate. If this does not appear at first, refiltering through the same paper will usually bring about the desired result. Standing overnight in a refrigerator will also increase the size of the precipitate particles and make filtration easier.

Biuret tests

Biuret test made on filtrates are negative or show so little color that one feels sure that the amount of protein material can have no significant influence on the findings. A comparison of the biuret reaction on the unfiltered and the filtered portion is sufficient to justify the conclusion that, even when this very faint trace of lavender or pink is seen, the amount of protein therein is certainly negligible.

Nonprotein nitrogenous constituents

Nonprotein nitrogen: The estimation of the total nitrogen on the filtrate is carried out by the usual Folin and Wu nesslerization method. It lies normally between 20 and 48 mg.%. In simple achylias the percentage is higher, up to 30 and 90 mg.%. In achylias complicated by carcinoma the figures have been as high as 60-150 mg.%.

Amino acids: The amount of amino acids was determined by the blood filtrate method of Folin. Because of the higher acidity, much more sodium carbonate must be used before the phenolphthalein will impart a permanent pink color to the solution.

Urea: Urea is determined by first estimating the amount of free ammonia plus the amount of ammonium liberated by the action of urease; from this result subtract the predetermined amount of ammonia.

Ammonia: Ammonia is determined by 2 methods: (1) unfiltered gastric juice, usually 5 ml., by the microaeration method of Folin and MacCallum and (2) upon the filtrate using the method of Folin and Wu.

Chapter 106

Duodenal content

STANLEY REITMAN
SAM FRANKEL

Examination of the duodenal contents, except for research purposes, is primarily indicated to determine whether or not there is an impaction of the common bile duct by gallstones. It indicates the presence or absence of inflammation and stones in the gallbladder. This absence of bile in duodenal contents may be due to a gallstone impacted in the duct or to a cancer at the head of the pancreas. If the bile contains pus cells, it is strongly suggestive of acute inflammation of the gallbladder. Crystals of cholesterol and granules of bile pigment may be found. If both of these are present in considerable amounts, it is presumptive evidence that there are stones in the gallbladder.

METHOD OF WITHDRAWAL

The duodenal fluid is obtained by means of a slender, flexible rubber tube, 3-4 mm. in outside diameter, with a perforated metal tip. It is marked with a series of black rings to indicate the distance from the incisor teeth to the cardia, to the pylorus, and to the duodenum.

If the pancreatic enzymes are to be studied, give a cup of bouillon 30 min. before the tube is introduced. In most of the duodenal contents examinations, however, a test meal is not used. The patient abstains from food for about 12 hr., taking only occasional sips of water. The tube is inserted into the stomach in the manner described for the stomach tube. Place the patient upon his right side with hips elevated 6 or 8 in. The movements of the stomach, aided by gravity, carry the metal tip through the pylorus into the duodenum, usually within 30-45 min. Fluid begins to drip from the free end of the tube soon after the metal tip has reached the stomach. If it does not do so, siphonage should be started by injecting a few ml. warm water. Collect the fluid in portions of about 5 or 10 ml. in a series of test tubes. The fluid that first appears is from the stomach and may be recognized by its acid reaction. When the tube enters the duodenum, the fluid becomes slightly alkaline and is usually clear, light yellow or colorless, and viscid. If one is in doubt as to whether or not the tube is still in the stomach, give the patient a few swallows of milk and aspirate a portion of the fluid through the tube. If the milk appears, the tube is still in the stomach. If the flow should at any time be interrupted, inject a few ml. warm water. If, after the tube is in the duodenum, the fluid is somewhat cloudy and opalescent, less alkaline, and less viscid, this is the result of admixture with stomach fluid which may have passed through the pylorus. Such fluid is to be discarded.

Examination of duodenal fluid should be done as soon after withdrawal as possible since many of its characteristics quickly change. **Normally** it is clear, colorless or light yellow, distinctly viscid, and slightly alkaline. Admixture of acid gastric juice causes it to become somewhat cloudy and opalescent. Cloudiness due to bacteria and pus cells may be present in inflammation of the duodenum or biliary passages.

LYON-MELTZER METHOD OF DUODENAL DRAINAGE[10]

Drainage of the gallbladder by inducing a relaxation of the sphincter of Oddi, through the introduction of a 25% solution of magnesium sulfate, has attracted a great deal of attention. Lyon claims that by adhering closely to his technic many cases of early biliary tract disease can be diagnosed from the examination of the duodenal contents. It has been proved, however, that the so-called A-, B-, and C-bile, presumably originating from three different parts of the biliary tract, have no clinical significance. (The A-bile is supposed to originate in the common duct; the B-bile, a dark viscous bile, in the gallbladder, and the C-bile, a golden yellow bile, in the hepatic duct.) Lintz reported a case that has dealt quite a blow to the A-, B-, and C-bile contention. His patient had congenital absence of the gallbladder and yet, upon injection of magnesium sulfate the 3 typical specimens of bile, including the dark B-bile, which is supposed to come from the gallbladder, appeared. Nevertheless, a careful microscopic examination of duodenal sediments may be of diagnostic value.

According to Jones, bile-stained leukocytes and epithelial cells, when found in any quantity, indicate an inflammatory condition of the biliary tract. It is not possible, though, to determine from the type of cell found in the duodenal sediments the exact level of the inflammatory process. Too much importance should not be attributed to the finding of these cells; they should be regarded merely as confirmatory evidence.

The presence of many crystals of cholesterin, bilirubin, and calcium bilirubinate may be of diagnostic value in cholelithiasis. It has been claimed by Bockus and associates that the finding of cholesterol crystals and bilirubin calcium pigment in the same bile is pathognomonic of cholelithiasis.

CHEMICAL EXAMINATION OF DUODENAL CONTENTS

Enzymes

Estimation of the pancreatic enzymes, amylopsin, steapsin, and trypsin, in duodenal contents gives the same information as their estimation in feces but is more reliable. Absence of or great decrease in the quantity of one or all of these enzymes points to deficiency of the pancreatic secretion or occlusion of the pancreatic duct. Pancreatic fluid from a fistulous tract shows little or no proteolytic activity. The following methods, although not absolutely accurate, give important clinical information.

Amylopsin (pancreatic amylase): Amylopsin should be studied along with amylase of urine and blood (p. 111). Carry out the technic for amylase in feces but use pure duodenal fluid, 5 ml. 1% starch, and bring the volume in each tube to 7 ml. Express the amount of amylase in units (the number of ml. 1% starch digested by 1 ml. duodenal contents). For example, if the digestion of starch is complete in the tube containing 0.2 ml. duodenal fluid, 25 units amylase are present. According to Myers and Fine, the average is about 40 units, with extremes of 5 and 200 units in different specimens.

Steapsin (pancreatic lipase)—Myers-Fine:

1. Place 1 ml. duodenal fluid in each of 2 test tubes.
2. Boil the contents of 1 tube to destroy enzyme activity.
3. Add to each tube 1 ml. neutral ethylbutyrate, 10 ml. distilled water, and a few drops of toluol.
4. Incubate at 38°-40° C. for 24 hr., shaking occasionally. If lipase is present, the ethylbutyrate will be split, the resulting fatty acid giving an acid reaction in the tube that was not boiled.
5. Add 2 drops phenolphthalein indicator to each.
6. Titrate to a pink color with N/20 sodium hydroxide.

The index of lipolytic activity is the difference in the number of ml. N/20 sodium hydroxide required for the 2 tubes. According to Myers and Fine, this is 0.3-4.3 ml., averaging 1.5 to 2.

Trypsin:

1. If the fluid is acid or neutral, render it slightly alkaline with a weak solution of sodium carbonate.
2. To 5 ml. fluid in a test tube add 1 gm. fibrin and a few drops of toluol.
3. Incubate at 38°-40° C. for 24-48 hr. Tyrosin and tryptophan can be found if trypsin is present. Neutralize the fluid and examine microscopically for tyrosine crystals. Test for tryptophan by adding very dilute bromine water a drop at a time. If tryptophan is present, a reddish violet color develops, which disappears upon the further addition of bromine.

Amylase:

1. To 10 ml. 1% solution of freshly prepared starch add 1 ml. pancreatic juice and 1 ml. toluol.
2. Allow the mixture to digest at 40° C. until duplicates no longer show blue with iodine solution. Place the bottles in boiling water to stop digestion at the same moment.
3. Dilute the contents to 50 ml.
4. Titrate from a buret against 10 ml. of boiling Fehling solution, using acetic acid and potassium ferrocyanide to determine the end point.
5. Ascertain the number of ml. of digestive mixture required to reduce 10 ml. Fehling solu-

tion, which is equivalent to 0.05 gm. dextrose, or its equivalent of maltose.

6. If the amount of sugar in the digestive mixture is less than that required to reduce the copper, add a standard dextrose solution and compute the amount of dextrose equivalent in the juice used.

Amylase values are reported as the number of ml. of the digestive mixture, which, when made up to 50 ml., would be equivalent to 0.05 gm. glucose. Normal values are given above.

Lipase:

1. Place in a bottle 1 ml. pancreatic juice.
2. Add 10 ml. distilled water, 1 ml. neutral ethyl butyrate, and 1 ml. toluol.
3. Allow mixture to digest at 40° C.
4. After 24 hr. determine the amount of acidity developed, using N/20 sodium hydroxide aqueous solution, with phenolphthalein indicator. From the amount of sodium hydroxide used subtract the amount of acidity, if any, in the duplicates containing 1 ml. juice plus 10 ml. water and 1 ml. toluol.
5. Shake the digestive mixture 10 times during the 24 hr. period.

Express the lipase values as the amount of N/20 sodium hydroxide necessary to neutralize the acidity developed. Normal values are given on p. 1923.

Alkali-protease (trypsin):

1. Dissolve 10 gm. gelatin in 100 ml. 1% solution of sodium fluoride and color with methyl violet.
2. Draw this solution into capillary tubing 1 mm. in diameter.
3. Quickly place the tubing under cold water to solidify the gelatin.
4. Cut the tubing into 2 cm. lengths.
5. Place 10 ml. juice in a small bottle.
6. Close with a perforated cork through which the gelatin tubes can be inserted.
7. Keep the digestions at room temperature for 48 hr. to determine the alkali-protease.
8. Add 1 ml. toluol to each digestive mixture and enough 0.2% sodium carbonate solution to make the mixture alkaline in reaction.

Express the alkali-protease (trypsin) values in the number of mm. gelatin digested. **Normal value** is 25-50.

Quantitative methods of determining pancreatic enzymes—Einhorn[11]

Principle: Agar tubes, mixed with albumin, starch, or fat, allow the enzyme action to take place in them by osmosis. If the test substances added are colored with indicators, which undergo a change when acted upon, it is easy to ascertain the presence of the enzymes and also to estimate their approximate activities by the volume of tube changes given in mm.

Starch tubes:

Agar powder	2.5 gm.
Starch	5 ml.
Distilled water up to . . .	100 ml.

1. Rub the starch and agar in a mortar with sufficient water to make a smooth paste; then add the rest of the water.
2. Place in a flask and heat to boiling point.
3. Draw by suction into a capillary glass tube, inside diameter 1.5 mm., which has previously been warmed in the flame. Allow tube and contents to cool. Cut into 2 cm. lengths. Seal at each end with melted paraffin.

Olive oil tubes:

Olive oil	25 ml.
Agar powder	2 gm.

Aqueous solution of Nile blue sulfate (1:2000), sufficient to make 100 ml.

1. Rub the olive oil and agar together.
2. Add sufficient water to make a thin paste.
3. Add the Nile blue sulfate solution to make the balance of 100 ml.
4. Then proceed as with the starch tubes.

Hemoglobin tubes:

Hemoglobin powder . . .	1 gm.
Agar powder	2.5 gm.
Distilled water sufficient to make	100 ml.

1. Rub the hemoglobin with about 10 ml. water until it is smooth; then add the agar powder and the balance of the water.
2. Then proceed as with the starch tubes.

Keep the agar tubes on ice until ready to use. They retain their efficiency for 4-6 wk., when they begin to deteriorate; they should not be used when they become dry.

Procedure:

1. Use one each of the starch, olive oil, and hemoglobin tubes.
2. Scrape off the paraffin at both ends.
3. Put vertically into a small bottle containing about 0.5 ml. duodenal secretion.
4. Add 1-2 drops of toluol and keep the bottle at 37° C. for from 16-24 hr.

Examine as follows: Take the agar tubes from the bottle, wipe the outside, and inspect. Hemoglobin tubes show a change of appearance due to action of trypsin, with a clearing up of the end part, becoming more or less transparent. Olive oil tubes manifest a bluish appearance at the end because steapsin activity splits the oil into fatty acids, which affect the indicator. Measure the lengths of transparency in the hemoglobin tube and of the bluish color in the oil tube in mm. Examine the starch tube by pushing out the agar column and dipping it into a weak iodine solution. The portion of starch column remaining colorless indicates the part changed by the amylopsin activity into sugar. This is expressed in mm. lengths.

By these tests the hemoglobin serves to gauge the trypsin activity, the olive oil measures the steapsin activity, and the starch measures the amylopsin activity.

Bilirubin

Bile pigment is usually present in the duodenal contents in sufficient amount to impart a tinge of yellow to the fluid. The depth of color is a useful guide to the amount present. Report as +, ++, +++, i.e., a small, moderate, or large amount, respectively. If bile is present, the diagnosis of complete obstruction of the hepatic or common bile duct is ruled out. Its absence does not exclude the possibility of bile reaching the duodenum at some time.

Urobilin

Urobilin is a derivative of bilirubin. It is doubtful whether or not the former idea of its identity with hydrobilirubin is correct. Urobilin is normally found in the duodenal contents just as it is in urine.

When we find an increase of urobilinogen and urobilin in the duodenal contents, it has the same significance as an increase of these substances in the stool. The amount of these substances in the feces and duodenal content reflects the extent of blood destruction. In other words, they are extremely important in the diagnosis of the hemolytic anemias. In the study of anemias in general the estimation of urobilin, when possible, is interesting but not necessarily important.

The most satisfactory clinical method for urobilin is that of Wilbur and Addis, which Schneider applies to the duodenal contents as follows.

Procedure:

1. To 10 ml. duodenal contents add 10 ml. saturated solution of zinc acetate. Shake well and filter.
2. To 10 ml. filtrate add 1 ml. Ehrlich reagent (p. 1827).
3. Mix and let stand in a dark place for 15 min.
4. Examine with a spectroscope and dilute with 60% alcohol until the bands of both urobilin and urobilinogen have disappeared exactly as is described for urobilin in feces.

Calculate the dilution value for 1000 ml. duodenal contents, remembering that the filtrate used represented 5 ml. duodenal fluid. If, for example, the urobilin and urobilinogen bands disappeared when the 10 ml. filtrate were diluted to 80 ml. and to 40 ml., respectively, then the dilution value of 5 ml. duodenal fluid is 16 for urobilin and 8 for urobilinogen; and for 1000 ml. it would be 200 times this, or 3200 and 1600, with a total dilution value of 4800.

Schneider found that the maximum value for healthy medical students is about 1000 dilutions and that urobilinogen is never present. In pernicious anemia and hemolytic jaundice urobilinogen is generally present, the total dilution value usually reaching 3000-5000. Giffin and associates found a striking decrease following splenectomy, particularly in pernicious anemia.

Schneider used these figures in the following formula, which aims to express the relation between blood regeneration and blood destruction in the form of an index number which he designated as the H-H (hematopoietic-hemolytic) Index:

$$\frac{Z + Y}{6} = \text{H-H index}$$

Z representing the total duodenal urobilin-dilution value in thousands and Y the red cell count in millions. Under normal conditions (urobilin dilutions 1000, red cell count 5,000,000) the index is 1. When hemolysis is very active, it will be above 1 unless counterbalanced by very deficient blood formation. When blood regeneration fails, the index will be below 1. Such an index is artificial and arbitrary but is useful in stressing the importance of considering the ratio of blood formation to blood destruction in the course of an anemia.

Fractional examination of duodenal contents[12]

Einhorn recommends a test meal of one cup of beef broth, usually made by putting one bouillon cube in a cup of hot water. The duodenal contents are examined first in the fasting condition, then one cup of buillon is given by mouth, and the duodenal contents are aspirated every ½ hr. for a period of 2 hr. This is carried out as follows: Give patient the duodenal tube in the evening after a light supper. Note the length of tube which has entered the buccal cavity before retiring. The tube used is that recommended by Einhorn with 3 marks, mark I, mark II, and mark III. Mark III should have reached the mouth; if not, the patient is given milk or water and the tube in this way made to go in beyond the III mark before going to sleep. Attach the tube, lying outside the mouth, with a piece of adhesive tape to the cheek. By early morning the capsule end of the tube has usually reached the duodenum.

Einhorn states that in normal patients there was an alkalinity of the duodenal contents of 30, whereas ½ hr. after the bouillon test meal it was 10, then it rose to 20, 25, and ultimately to 30. The enzyme activities were strongest in the fasting condition, whereas they were weakest ½ hr. after the test meal, to increase in strength later on. In pathologic cases the degree of alkalinity of the duodenal contents appeared to be dependent to a certain extent upon the state of gastric secretion. Einhorn states that cases of hyperchlorhydria showed a lowered alkalinity whereas cases of subacidity manifested an increased alkalinity. Other factors play a role, chief of which are pancreatic and duodenal juices and the bile. He differentiates 4 types of alkalinity curves: **type I**—decrease of the alkalinity during the first ½ hr. after the bouillon test meal, then a steady increase; **type II**—increase of alkalinity between ½ and 1 hr. after the bouillon, then slight decrease; **type III**—increase of alkalinity during the first ½ hr. after the bouillon, then slight decrease up to 1 hr., then again increase to 1½ hr., thereupon again a decrease; **type IV**—at first a steady increase of alkalinity up to 1½ hr. after the bouillon test meal, then an abrupt descent of the curve, so that at 2 hr. after the bouillon the contents are acid instead of alkaline.

Einhorn[13] gives the following 4 types of reaction in **peptic ulcers:**

1. Duodenal contents are always alkaline in the fasting state as well as at any time after the bouillon test meal.
2. The duodenal contents are alkaline in the fasting state and at times acid after the bouillon test meal.
3. The duodenal contents are acid in the fasting condition and at certain periods after the bouillon test meal.
4. The duodenal contents are always acid in the fasting state as well as at any time after the bouillon test meal.

Type I represents the normal. In a number of peptic ulcers the abnormal types (II, III, and IV) were found. The severity of the disturbance is least in type II, greater in type III, and greatest in type IV.

MICROSCOPIC EXAMINATION OF DUODENAL CONTENTS

Unless the duodenal fluid is examined within a few minutes after it is secured, the cellular elements may be damaged or destroyed by the enzymatic action. The method is the same as for fresh urine. Normally only an ocasional leukocyte or epithelial cell can be found. Pathologically these may be present in increased numbers, but no definite diagnostic inferences can be drawn. A great excess of pus cells suggests inflammation of the duodenum or biliary tract. *Strongyloides stercoralis* and *Giardia lamblia* have been found, sometimes in great numbers. Cystic and vegetative forms of *Entamoeba histolytica* have also been found, and in such cases infection of the liver or bile passages is inferred.

BACTERIOLOGIC EXAMINATION

At the present time very little of clinical value can be learned from a bacteriologic study. Normally the fluid is sterile or contains only a few gram-positive cocci. Bacteria seen in the direct microscopic examination are mostly dead.

For bacteriologic examination the duodenal fluid is obtained in the usual way, with the following precautions to prevent contamination:

Sterilize the tube by boiling. Slip over the metal tip a gelatin capsule which has been soaked in alcohol for several days. Dip the gelatin-covered tip in thin shellac several times, letting it dry after each coating. Introduce the tube in the usual way. When it has entered the duodenum, the gelatin bag may be removed by forcing in a little air or a few ml. sterile water.

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Section Three

CEREBROSPINAL FLUID

Chapter 107

Cerebrospinal fluid

JOHN D. BAUER

The examination of spinal fluid is a very important procedure in connection with many medical and surgical cases. For the method of withdrawing spinal fluid from the living subject, we are indebted to Heinrich Quincke who first treated 2 cases of hydrocephalus by means of withdrawal of the spinal fluid. In 1893 Lichtheim called attention to spinal puncture as a valuable aid in diagnosis, and in 1895 Fubringer reported 107 punctures in 80 cases. Since that time spinal puncture has been generally adopted as a routine procedure for diagnostic and therapeutic purposes.

The ventricles of the brain which contain fluid slightly different in composition from that of the spinal fluid were first tapped by Middeldorpf in 1856. Occipitoatlantoid drainage of the cisterna magna in man was first performed by Parker in 1893. Cistern puncture has been developed mainly in this country. In 1919 Wegeforth, Ayer, and Essick performed the operation in man, using a needle for the puncture. Sicard in 1923 was the first to introduce Lipiodol into the cistern as a roentgenographic aid in the diagnosis of cord tumors.

The cerebrospinal fluid is contained in the ventricles of the brain and in the subarachnoid space of the brain and cord. There are 5 ventricles, 2 lateral, and the third, fourth, and fifth. They are intercommunicating. The cerebrospinal fluid in the ventricles of the brain communicates with the fluid in the other ventricles through the various apertures connecting them. The only exception to this is the fifth ventricle which contains very little or no fluid. The fluid in the lateral ventricles flows into the third through the foramina of Monro; the fluid in the third communicates with that in the fourth by means of the aqueduct of Sylvius. The cerebrospinal fluid of the subarachnoid space communicates with the fluid in the ventricles of the brain through the foramen of Magendie, which opens into the posterior part, and the 2 foramina of Luschka, which open into the lateral recesses of the fourth ventricle, and through numerous other apertures which open into the lateral ventricles.

In order to understand the diagnostic features of cerebrospinal fluid, something must be said of the physiology of this fluid. No one has yet determined the rate of formation under normal conditions. Equally uncertain are the data on the rate of absorption of the spinal fluid, but it has been shown that absorption probably occurs through the perivascular lymphatics directly into the blood stream.

The function of the cerebrospinal fluid is mechanical. The fluid forms a water bed around the cord and brain and prevents jarring from external trauma. It also equalizes the pressure between the brain and the cord. It is probable that the chorioid plexus, which is in the ventricles of the brain, is responsible for the secretion of the cerebrospinal fluid.

CHARACTERISTICS OF CEREBROSPINAL FLUID

Amount: The amount of fluid normally present in the ventricles and in the subarachnoid space varies according to different authors from 60 to 150 ml.

Color: Normal cerebrospinal fluid is clear and colorless, provided no blood has been removed with it during the puncture.

A very interesting condition which is called xanthochromia may sometimes be found. In this condition the spinal fluid is a clear yellow color with rapid and marked coagulation, large amounts of globulin, and no blood pigments by chemical tests. It is generally the result of compression by a tumor of the cord or vertebrae with interruption to the flow of cerebrospinal fluid and formation of a pocket of greater or lesser extent in which the fluid stagnates. Xanthochromia also follows old brain hemorrhage and is thought to be due to bilirubin bound to albumin.

Transparency: Normal spinal fluid is clear. In acute meningitic infections the fluid may vary from slight cloudiness to almost pure pus. It is absolutely clear in tuberculous meningitis.

Pellicle formation: In many acute cases of meningitis a pellicle or a net of fibrin and blood cells forms in the fluid on standing. In suppurative meningitis the pellicle forms in a very short time; in tuberculous meningitis it takes 12-24 hr. to form. Pellicles have been observed in poliomyelitis and in general paresis only rarely. The pellicle is formed by the separation of the heavier elements of the cerebrospinal fluid, so that microscopically one sees in the pellicle many leukocytes on a fibrinous background. Fig. 685 shows the forms of pellicle in various diseases as observed early, before autolysis takes place. The pellicle is dependent for its formation upon the presence of fibrinogen.

Lack of sediment: Normal fluid forms no pellicle or sediment. This lack of sediment is helpful in differentiating normal fluid from fluid in cases of meningitis in which a sediment is often present.

Specific gravity: The specific gravity varies according to different authors from 1.006 to 1.010.

Reaction: Reaction of cerebrospinal fluid is alkaline.

Levinson[1] states that the hydrogen ion concentration ranges from pH 7.4 to 7.6.

Total solids: Total solids vary between 1.200 and 0.876 gm./100 ml.

Calcium: Calcium content of the spinal

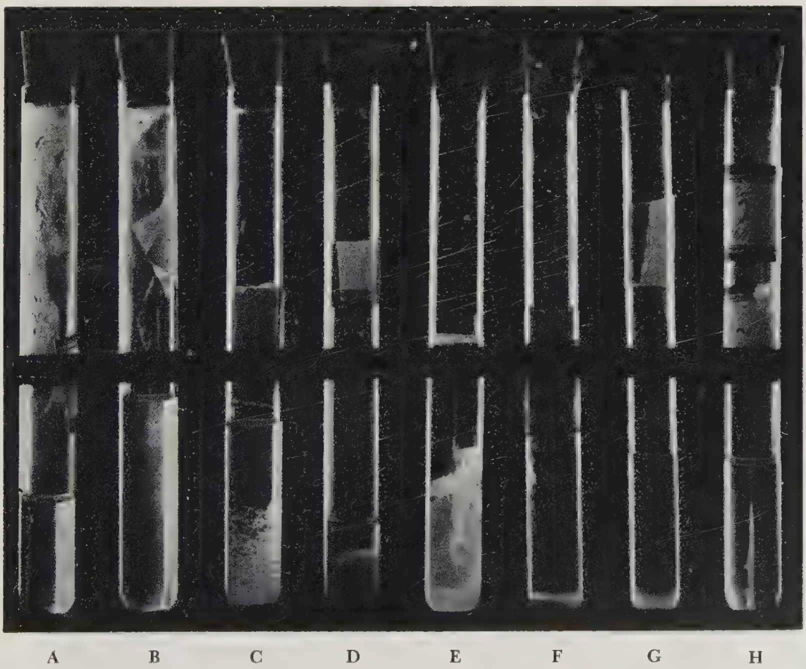

Fig. 685. Pellicle formation in meningitis. **A,** Normal. **B,** Meningococcus meningitis, fairly early. **C,** Meningococcus meningitis, advanced (sulfurlike granules). **D,** Meningococcus meningitis, late in disease. **E,** Meningococcus meningitis, severe, on road to recovery. **F,** Pneumococcus meningitis, fatal. **G,** Pneumococcus meningitis, fatal. **H,** Tuberculous meningitis. (From Levinson, A.: Cerebrospinal fluid in health and in disease, St. Louis, The C. V. Mosby Co.)

fluid varies from 2.3 to 2.6 mg.% (1.13-1.3 mEq./L.).

Magnesium: Magnesium content of spinal fluid is greater than that of blood (0.82-1.08 mEq./L.).

Chlorides: Normal spinal fluid contains more chloride than does blood plasma. The normal value for adults is 123-133 mEq./L. spinal fluid. In children the normal range is 110-130 mEq./L.

In acute meningitis the amount of chloride may be reduced in adults to 105 mEq./L. and to 98 mEq./L. in children. In tuberculous meningitis still smaller amounts are found. A fall in plasma chlorides will produce a corresponding fall in C.S.F. chlorides. High values may be found in conditions in which the value for the plasma chloride is increased.

For quantitative determination of chloride in spinal fluid see p. 176.

CYTOLOGY OF SPINAL FLUID

Most writers are of the opinion that the normal cell count in spinal fluid varies from none to 2/c.mm. As a rule, we consider anything up to 5 normal.

Type of cells found: The cells of spinal fluid are leukocytes, principally small lymphocytes. The nucleus of the lymphocytes of normal spinal fluid is round or slightly oval, seldom irregular. Large lymphocytes occur very rarely. Polymorphonuclear or segmented leukocytes are usually absent in normal fluids. The origin of the cells in normal spinal fluid has not been fully determined.

Method of cell counting in spinal fluid

The cell count should be made as soon after withdrawal as possible. Always shake the tube before diluting the spinal fluid.

Reagent:

1. Spinal fluid counting solution (Tuerk solution).
   Glacial acetic acid . . . . . 1 ml.
   Distilled water . . . . 99 ml.
   (a) Add a few drops of alcoholic gentian violet to color.
   (b) Filter several times.

Procedure (with any counting chamber):

Diluting spinal fluid:

1. Draw the spinal fluid counting solution to the 0.5 mark and the spinal fluid to mark 11 in a white counting pipet, making a dilution of the spinal fluid of 19:20.
2. Mix thoroughly.

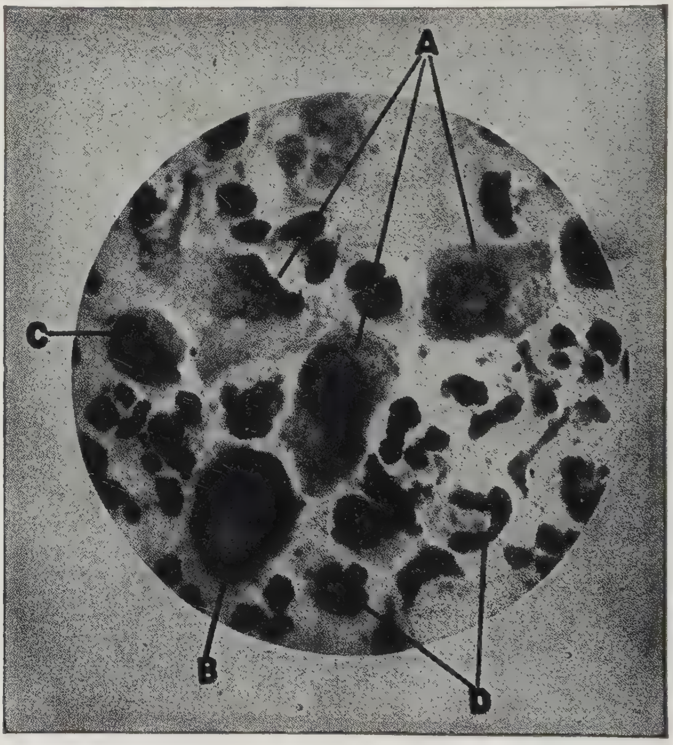

Fig. 686. Photomicrograph showing types of cells in pathologic cerebrospinal fluid. **A,** Desquamated endothelial cells. **B,** Monocyte which has engulfed a polymorphonuclear leukocyte. **C,** Large mononuclear cell. **D,** Polymorphonuclear leukocytes. (×1000.) (From Levinson, A.: Cerebrospinal fluid in health and in disease, St. Louis, The C. V. Mosby Co.)

Filling counting chamber:

1. Blow out 2 or 3 drops of the diluted spinal fluid before filling the counting chamber. Place the cover glass over the ruled area.
2. Allow the diluted spinal fluid to run under the cover glass so that it exactly fills the space between the cover glass and the counting chamber.

Making count:

1. Focus under the low-power dry objective without the Abbé condenser.
2. Count all the cells in the entire ruled area which represents 9 fields.
3. Divide by 9 and multiply by 10 to obtain the number of cells/c.mm.
4. Make similar counts on 2 more drops of diluted spinal fluid.
5. Average the 3 counts for the report.
6. To correct for the slight dilution of the spinal fluid, divide the final result by 19 and multiply by 20. Do not report a fraction of a cell.

Example:

1st drop	2nd drop	3rd drop
9 | 18	9 | 20.0	9 | 18
2	2.2	2
10	10	10
20	22.	20
22		
20		
3 | 62		
19 | 20.6		

1.08 × 20 = 21.6 or 22 cells/c.mm. spinal fluid

Normal: 0-5 cells/c.mm. (see below).

Counting of spinal fluid contaminated by blood

The problem of whether or not an accurate cell count can be made of bloody spinal fluid may be answered in the following words—to date no reliable or absolutely accurate method can be recommended for estimation of pleocytosis in the cerebrospinal fluid if the fluid is contaminated by blood. An attempt has been made to do this by staining the fluid lightly with Unna polychrome methylene blue and calculating all of the red cells and all of the white cells present in 1 c.mm. Correction for the number of white cells present in the fluid as a result of the blood can then be made on the basis of the red and white blood count in the peripheral vessels. According to Merritt,[2] "while this method is not absolutely accurate there is usually no trouble determining a pleocytosis in the cerebrospinal fluid of twenty-five or more cells."

Making the differential count of spinal fluid

This count can be made if there is a total blood cell count of over 500 cells/c.mm. If the cell count is below this, the sediment may be procured by centrifuging the fluid.

Technic of procuring the cells is to discard the supernatant fluid and spread the cells on a glass slide. In either case, whether the direct count or count of this centrifuged specimen is to be made, proceed in exactly the same manner in which a differential count of the leukocytes in blood is made.

Interpretation: As stated above, the normal cell count in the spinal fluid is 0-5 cells/c.mm. in adults.

Increased cell count is due to inflammatory lesion of the brain, spinal cord, or meninges. An increased count is found in cerebral tumors. An acute process will show an increase in the segmented neutrophils. In chronic infections and toxic conditions there is usually an increase in lymphocytes. A slight increase, say 10-100, especially of the lymphocytes, occurs in early tuberculous meningitis, neurosyphilis, anterior poliomyelitis, encephalitis lethargica, and multiple sclerosis. As many as 1000 cells, principally lymphocytes, have been found in late tuberculous meningitis, poliomyelitis, and acute syphilitic meningitis. Naturally, over 1000 cells are found in purulent meningitis and brain abscess.. There is no increase in cells to any extent in uremic or diabetic coma.

The **Papanicolaou technic** in conjunction with the Millipore filter* has also been applied to the examination of the cerebrospinal fluid for tumor cells. In primary tumors of the brain the results are disappointing, but they are somewhat improved in metastatic tumors with meningeal involvement.[3]

TESTS FOR PROTEIN

These tests are commonly called tests for globulin, but they are really tests for protein since the methods used depend upon the precipitation of both albumin and globulin.

Normal concentration of cerebrospinal protein is between 15 and 40 mg./100 ml. fluid. In inflammatory conditions of the meninges the choroid plexus, which is the barrier of the passage of the proteins, breaks down and permits these to enter the spinal fluid. In some other conditions such as typhoid fever, uremia, and convulsive states, elevated proteins are found. The increase in the proteins of the spinal fluid in suppurative diseases of the meninges is due to the accumulation of the protein of the exudate. In acute suppurative meningitis protein concentration values may be as much as 1300 mg./100 ml. Tuberculous meningitis shows values between 200 and 1000 mg. In tetanus there is an increase in the protein concentration, and an increase has been noted shortly after epileptic seizures. In such syphilitic diseases as paresis, tabes dorsalis, and other forms of cerebrospinal syphilis, values between 20 and 200 mg. have been reported. In epidemic encephalitis, protein concentrations vary between 50 and 300 mg./100 ml. In cerebral hemorrhage, thrombosis, or embolism there is an increased protein content. There are increased protein contents in brain abscesses and brain tumors and also in multiple sclerosis. In the disease known as the Froin syndrome, in which there is complete obstruction of the meninges of the spinal cord, there is a difference noted in the protein concentration above and below the point of obstruction. For instance, a lumbar puncture may show in such a case as much as 1000 mg./100 ml. (due to absorption of water below obstruction), and fluid taken from a cisternal puncture may show practically normal values. In myxedema, protein varies between 30 and 220 mg. Table 367 shows the changes in the pathologic conditions in respect to protein concentrations. It is important to note that the normal range varies according to the location from which the specimen is obtained.

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*Millipore Filter Corp., Bedford, Mass.

Table 367. *Cerebrospinal fluid proteins**

Condition	*Changes in spinal fluid protein to be expected*	*Concentrations observed (in mg./100 ml.)*
Normal	0	20-40
Pathologic		
Brain tumor or abscess	+	30-100
Cerebral hemorrhage†	+	20-220
Cerebral thrombosis†	+	20-140
Cerebrospinal syphilis†	+	20-160
Epilepsy	+	20-80
Epidemic encephalitis†	+	50-300
Froin syndrome†	++	100-2100
Meningitis		
Meningococcic†	++	150-750
Pneumococcic†	++	180-1300
Streptococcic†	++	150-900
Tuberculous†	+	50-200‡
Multiple sclerosis†	+	
Myxedema	+	30-220
Paresis†	+	30-190
Serous meningitis (from influenza, otitis media, mastoiditis, pneumonia, typhoid fever, uremia, etc.)	0 to +	20-90
Spasmophilia or tetany	+	
Tabes dorsalis†	+	40-200
Tetanus	+	
Uremic convulsions	+	

*From Andes, J. E., and Eaton, A. G.: Synopsis of applied pathological chemistry, St. Louis, 1941, The C. V. Mosby Co.

†Diseases in which the determination may be of clinical value.

‡Values as high as 1000 have been reported, but such concentrations are rarely found.

Normal:

Ventricular fluid .	10-15 mg./100 ml.
Cisternal fluid . .	15-25 mg./100 ml.
Lumbar fluid . .	20-40 mg./100 ml.

The first test described below, the Nonne-Apelt or Ross-Jones test, is a test for globulin as are the Pandy and Noguchi tests.

Qualitative tests

Nonne-Apelt method (Ross-Jones)

Reagent:

1. Nonne-Apelt (Ross-Jones) reagent. Saturated solution of ammonium sulfate, filtered after saturation has been reached.

Procedure:

1. Place 1 ml. Nonne-Apelt (Ross-Jones) reagent in a test tube.
2. Stratify 1 ml. spinal fluid on the reagent.

A white ring at the point of contact indicates the presence of globulin. If the spinal fluid contains blood, the test should not be made.

Pandy method

Reagent:

1. Pandy reagent.
   (a) Dissolve 10 gm. carbolic acid crystals in 150 ml. distilled water.
   (b) Place in the incubator and shake frequently for several days. Filter. The reagent must not become turbid upon shaking. It may become pink.

Procedure:

1. Place 1 ml. Pandy reagent in a test tube.
2. Add a few drops of spinal fluid and shake.

A turbidity develops if globulin is present.

Noguchi method

This test is so delicate that it often detects globulin in normal spinal fluids.

Reagent:

Butyric acid	10 parts
Sodium chloride, 0.9% . . .	90 parts

Procedure:

1. In a small test tube place 0.5 ml. Noguchi reagent.
2. Add 0.1 ml. spinal fluid.
3. Bring to a boil over a flame.
4. Add 0.1 ml. N/1 NaOH.

A flocculent precipitate appears in a few minutes if globulin is present in large quantities. The precipitate may take a few hours to form if the quantity of globulin is low.

Normal spinal fluids show a slight opacity. Traces of proteins are normally present in spinal fluid. A great increase occurs in acute inflammations in tuberculous meningitis, and in syphilitic affections.

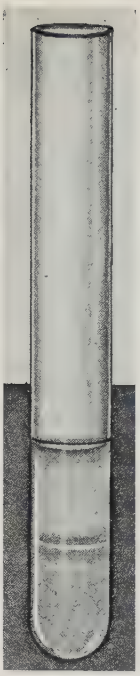

Fig. 687. Nonne-Apelt test for globulin in spinal fluid, positive reaction.

The chief protein is globulin, tests for which are outlined above. Because of the presence of serum globulin in blood, a globulin test is valueless if the spinal fluid is contaminated with blood.

Alcohol test

A drop of cerebrospinal fluid is added to a test tube containing 0.5 ml. 95% alcohol. A very slight turbidity is normal, but a heavy white cloud or flocculation indicates a high protein content.

Lange colloidal gold test

This test is based on changes in spinal fluid protein. The method that is recommended for the preparation of Lange colloidal gold solution is that suggested by Levine.[5] He had previously[6] described a method for this preparation using gold chloride, potassium carbonate, and formaldehyde. At that time he suggested the use of triply distilled water and a block-tin or resistant-glass distilling assembly. In 1934 Borowskaja[7] found that sodium citrate could be used in the preparation of gold sol instead of the potassium carbonate and the formaldehyde. She found, further, that sodium citrate stabilized the reaction sufficiently so that once-distilled water could be employed with safety. Levine made a study of Borowskaja's reaction and found that the time which it took for the reaction to reach its completion varied in duration from 5-30 min. and occasionally even longer, and that the sensitivity of the final sol to precipitation by positive spinal fluids varied accordingly. But he found that by the addition of a few drops of a fresh solution of 3% hydrogen peroxide instead of bubbling air or oxygen through the hot water, the reaction began immediately and reached completion within a few seconds. The method which is here advocated is that originated by Levine.

Glassware should be chemically cleaned. Although it is recommended that once-distilled water be adjusted to pH 7.2 to 7.4, this is not essential.

Preparation of colloidal gold:

1. To prepare 500 ml. gold sol the following formula is recommended:

Distilled water	460 ml.
Gold chloride, 1% solution .	5 ml.
Sodium citrate, 1% solution .	25 ml.
Hydrogen peroxide, fresh 3% solution, 3-5 drops, diluted in distilled water	10 ml.

2. Heat the distilled water to brisk boiling.
3. Remove from the hot plate.
4. Add the 5 ml. 1% gold chloride solution.
5. Shake for a few seconds.
6. Add the 25 ml. 1% sodium citrate solution.
7. Shake for 10-15 sec.
8. While shaking, add the 10 ml. distilled water which contains the 5 drops hydrogen peroxide. Continue shaking for a few seconds. Let the flask rest on the table until the reaction is completed. This usually takes ½-1 min.

The advantages of this method are resident in the fact that (1) it is not necessary to use triple distilled water; (2) the reaction of the water does not have to be adjusted; (3) this method can be used to make as little as 100 ml. and can be made with the same ease and with as high a degree of reliability as larger volumes. In Levine's experience, however, the reaction proceeds in a more regular manner in large volume manufacture, say, 500 or 1000 ml.

The colloidal gold reagent is commercially available.

Note: The prepared solution ordinarily requires no special treatment for neutrality. It is advisable, however, to test it with known normal and known paretic spinal fluids. It must give no reactions beyond the red-blue color with a known normal spinal fluid, and a typical paretic zone curve with the known paretic spinal fluid. The neutrality of the colloidal gold solution must be controlled; this may be carried out with alizarine red. Place 10 ml. colloidal gold fluid in a clean flask, add 1 or 2 drops 1% solution of alizarine red in 50% alcohol. Titrate with N/50 sodium hydroxide until neutral. With this solution of alizarine, acid reaction shows lemon yellow; neutral, brown red; alkaline, reddish purple. After estimating the quantity of acid or alkali necessary to neutralize the 10 ml. sample, add enough of one or the other to neutralize the entire amount of colloidal gold reagent that has been prepared.

A special set of glassware should be kept for the making of the reagent and the test. This glassware should be chemically cleaned, and care should be exercised in preventing contamination of the spinal fluid with dyes or chemicals before it is tested. Glassware should also be cleaned in 50% **aqua regia** (3 parts HCl, 1 part HNO_3, 4 parts water) to remove precipitated gold.

Glassware:

1. 1000 ml. Pyrex beaker, free from scratches.
2. 1000 ml. cylinder graduated in 10 ml.
3. 1000 ml. brown glass-stoppered bottle for storing the reagent.
4. 100 ml. cylinder graduated in 1 ml.
5. 25 ml. volumetric pipet.
6. 10 ml. pipet, graduated in 0.1 ml.
7. Two 1 ml. pipets, graduated in 0.01 ml.
8. Two 5 ml. volumetric pipets.
9. Several test tubes, kept especially for use in testing the reagent.
10. Eleven test tubes for the actual test, 1 cm. in diameter and 10 cm. long.

Procedure: Test tubes, 1 cm. in diameter and 10 cm. long, the same size as Wassermann test tubes, are needed for the test. In addition, two 1 ml. pipets, graduated in 0.1 ml. and one 5 ml. volumetric pipet are required.

1. Dilute physiologic saline to double volume for the test since a 0.4% sodium chloride solution is used.
2. Place the 11 test tubes in a Wassermann test tube rack.
3. Leave one space between the tenth and the eleventh tube. Tube 11 is a control over the glassware and the reagent. It receives no spinal fluid.
4. Place 1.8 ml. 0.4% sodium chloride solution in tube 1 and 1 ml. in each of the remaining tubes, including tube 11.
5. Add 0.2 ml. spinal fluid, free from blood cells, to tube 1.
6. Mix by drawing in and out of the pipet many times.
7. Withdraw 1 ml. of the mixture in tube 1 and place in tube 2. Mix the contents of tube 2 in the same manner; withdraw 1 ml. and place in tube 3. Mix the contents of this tube; withdraw 1 ml., and place in tube 4. Continue in this manner through tube 10.
8. After mixing the contents of tube 10, withdraw 1 ml. of the mixture and discard. Tube 11 receives no spinal fluid.
9. Place 5 ml. Lange colloidal gold in each of the 11 tubes.
10. Mix thoroughly and allow to stand at room temperature for 24 hr. Record the results according to Fig. 692 or record the numbers represented by the color changes in each tube according to Fig. 692. The color scale, with corresponding numbers, is as follows:

5 colorless
4 pale blue
3 blue
2 lilac or violet or purple
1 red blue or very slight change
0 red, no change in color of reagent

The **principle** upon which this test is based is as follows: if a cerebrospinal fluid is diluted in geometrical progression with a weak neutral electrolyte and 5 vol. clinical colloidal gold are added to each successive solution, a series of precipitation colors may be obtained which when ascribed numerical values from 0 to 5 and the values plotted as ordinates against the dilutions as abscissae will give a curve whose contour is characteristic for certain of the infectious diseases of the central nervous system.

The recording of the color values or ordinates is then plotted on coordinated paper against the dilutions or abscissae. Multiply the abscissae values by some convenient factor to elongate the curve formed by connecting the points in a continuous line. A convenient factor is 5/3.

The color of the colloidal gold is red. No change in the color of the reagent is therefore red, or "0" on the color scale. Complete precipitation of the gold is accompanied by a change in color of the reagent from red to colorless, the color changes between red and colorless depending on the amount of precipitation.

Interpretation: It has been generally believed ever since Lange brought out this reaction in 1912 that it is diagnostic of syphilis of the nervous system and particularly that various types of neurosyphilis could be distinguished by changes in this reaction. The weight of opinion at the present time, however, is to discard the terms "paretic," "cerebrospinal luetic," and "meningitic curves" and replace them by terms that indicate the tubes in which the greatest precipitation of the colloidal suspension occurs—"first-zone," "mid-zone," and "end-zone." This test indicates an abnormality in the protein content of the cerebrospinal fluid and does not necessarily have any particular diagnostic value. Kafka[8] and others have shown that the changes produced in colloidal suspensions of spinal fluids are related to the albumin-globulin ratio and to the total protein content of the fluid. In other words, the globulin fraction of the protein of the cerebrospinal fluid is most active in the precipitation of the colloidal suspension, whereas the albumin fraction has a protective action; this protective action depends not only on the albumin-globulin ratio but also on the total amount of protein in the fluid. A fluid with a high protein content and a high albumin-globulin ratio would produce no change in the colloidal suspension, but dilutions of it would cause a precipitation.

A normal spinal fluid results in no change of the colloidal gold suspension except possibly a slight change to lilac.

The first-zone curve is seen in cases of paresis but is not necessarily diagnostic of this condition because it has been seen in multiple sclerosis and other conditions (Fig. 692).

Text continued on p. 1940.

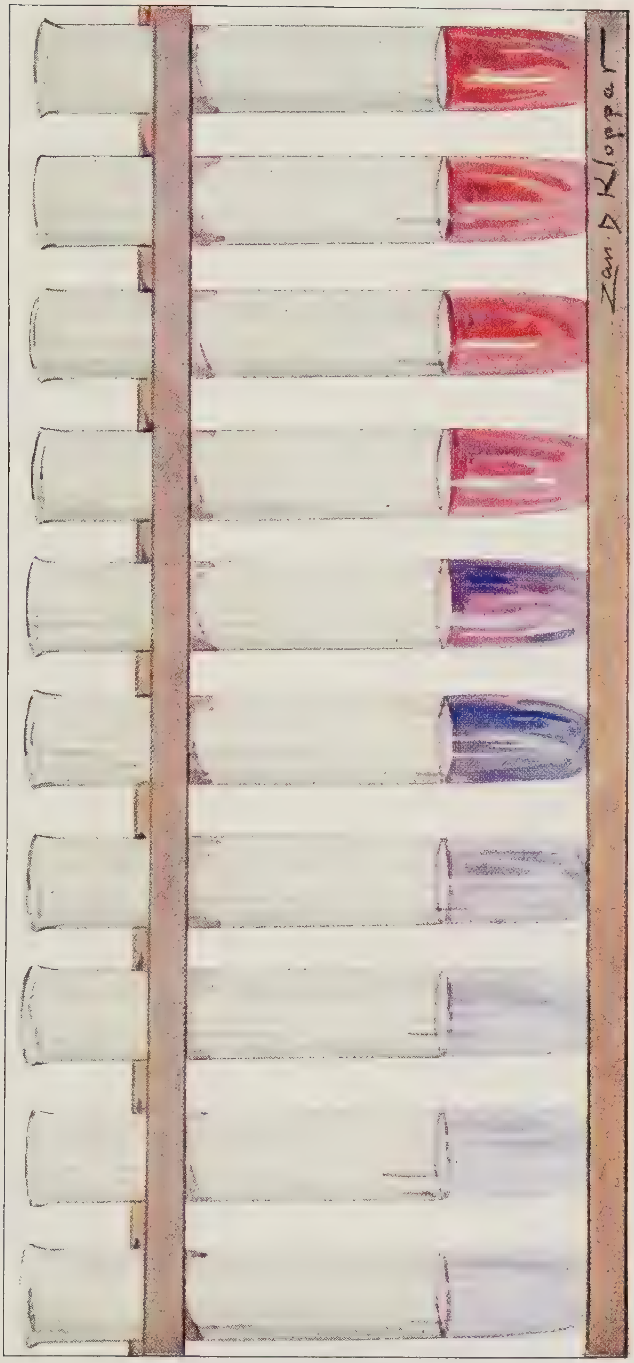

Fig. 688. Colloidal gold reaction in case of general paresis. (From Levinson, A.: Cerebrospinal fluid in health and in disease, St. Louis, The C. V. Mosby Co.)

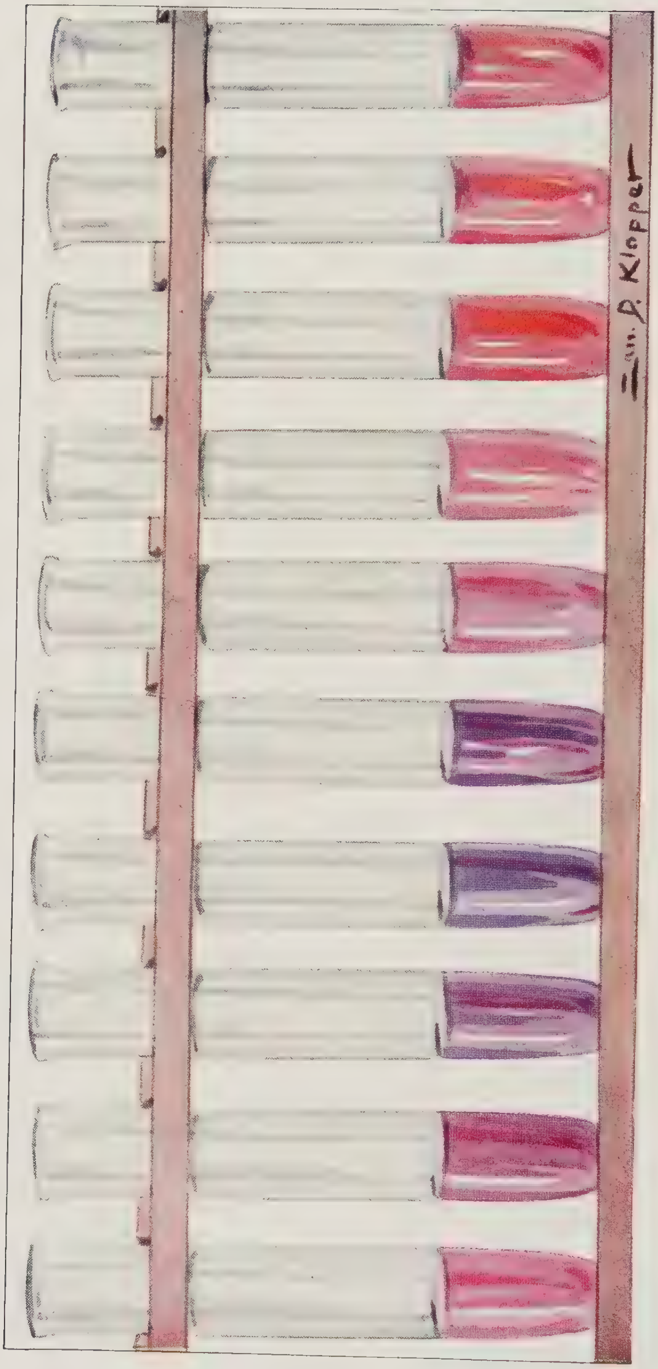

Fig. 689. Colloidal gold reaction in case of tabes dorsalis. (From Levinson, A.: Cerebrospinal fluid in health and in disease, St. Louis, The C. V. Mosby Co.)

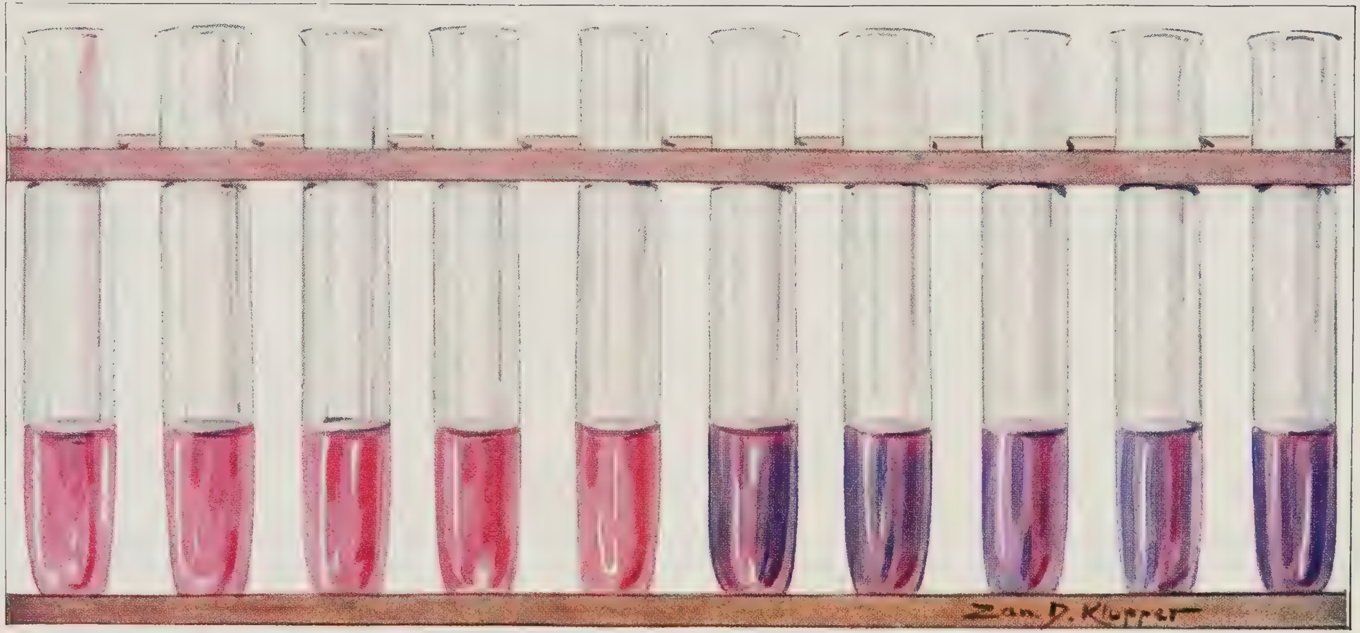

Fig. 690. Colloidal gold reaction in case of meningococcus meningitis. (From Levinson, A.: Cerebrospinal fluid in health and in disease, St. Louis, The C. V. Mosby Co.)

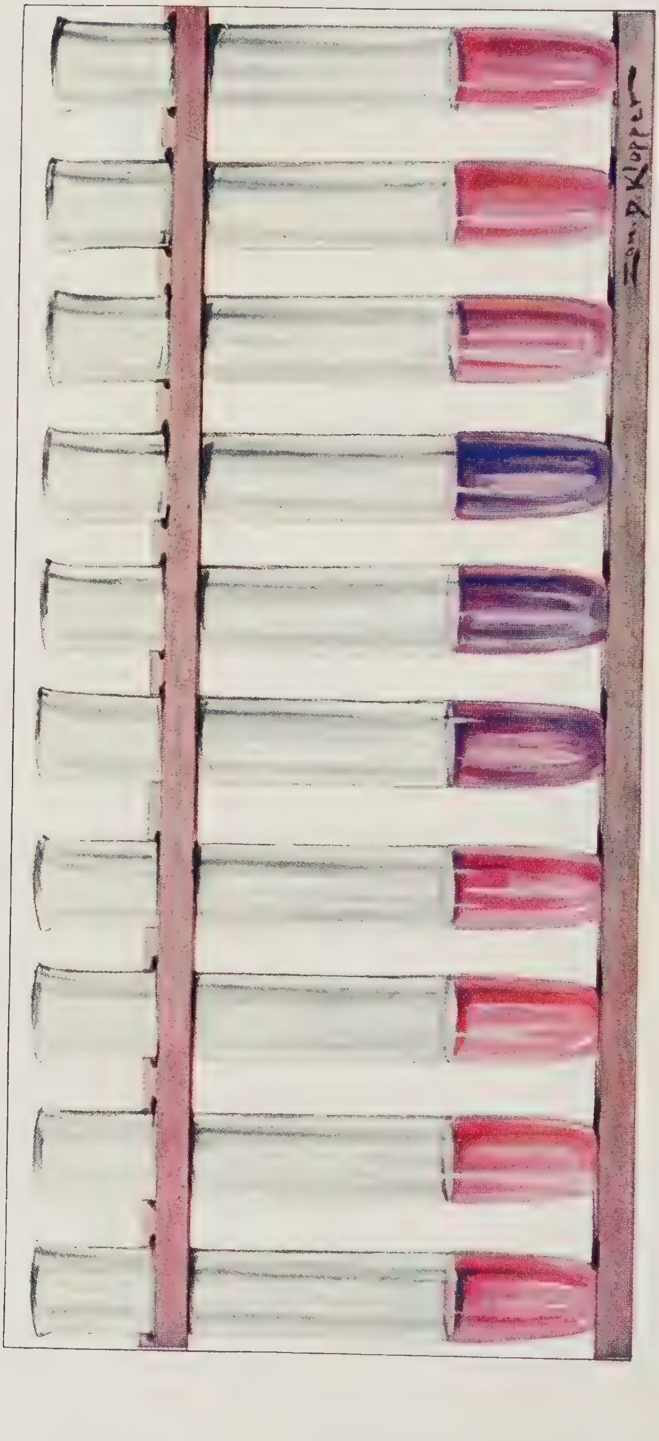

Fig. 691. Colloidal gold reaction in case of tuberculous meningitis. (From Levinson, A.: Cerebrospinal fluid in health and in disease, St. Louis, The C. V. Mosby Co.)

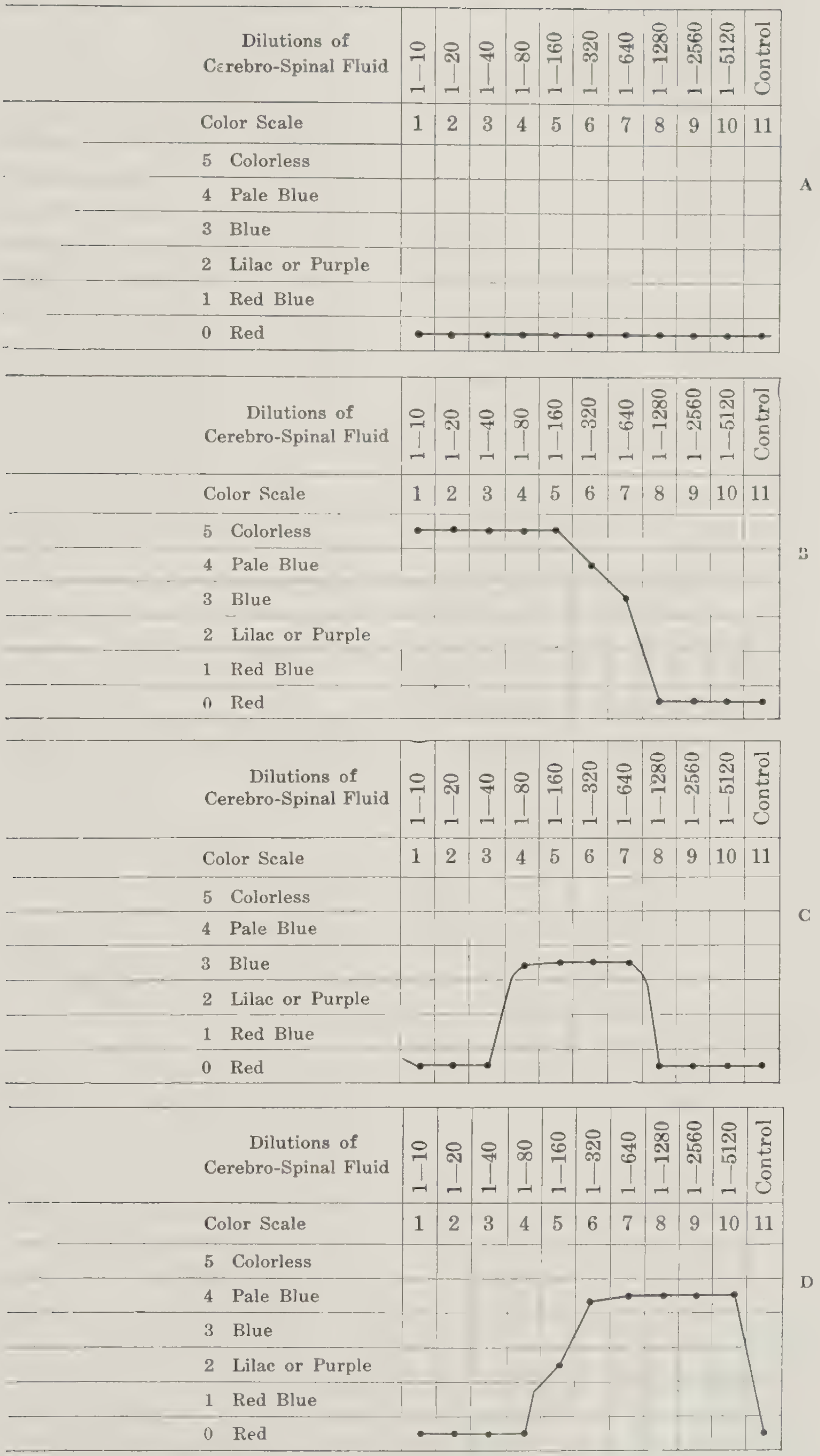

Fig. 692. Interpretation of results. **A,** Normal curve 0000000000. **B,** First-zone curve 5555543000. **C,** Mid-zone curve 0003333000. **D,** End-zone curve 0000244440.

The mid-zone curve (Fig. 692) may be found in any abnormal fluid and cannot be used as a specific indication of any particular type of cerebrospinal syphilis.

The end-zone curve (Fig. 692) was formerly called the meningitic curve but really means a fluid with a very high protein content with an especially high albumin-globulin ratio. It is naturally found in cases of acute purulent meningitis, subarachnoid block, and extravasation of blood in the subarachnoid space.

It is perfectly reasonable, therefore, to find changes in the colloidal gold test without any other signs of syphilis in the nervous system. Too much emphasis has been laid in the past on the specific value of this test alone as a differential diagnostic measure in cerebrospinal syphilis.

Precautions: It must be remembered that the colloidal gold solution contains only 1 part of gold in 20,000 parts of water, and therefore the presence of even a minute trace of contaminant may have a disastrous effect on the proper precipitation color. It was shown by Zsigmondy[9] that when 0.5 ml. 10% solution of sodium chloride is added to 5 ml. gold solution containing 0.25 mg. gold, more than 0.0025-0.005 mg. gelatin or 0.003-0.02 mg. alkali albumin[10] will prevent the change from red to violet.

Probably all the errors made in connection with this test are due either to inadvertent protection of the gold solution before or after use or to inadvertent precipitation afterward. One must be sure to prevent the intrusion of even traces of foreign disturbing substances into these solutions. The spinal fluid must be received into specially prepared test tubes which contain no traces of gelatin or albumin. Concerning the use of salt solution in this test, it is advisable to pour out the quantity that is going to be used from the stock bottle into a separate clean container so that repeated insertion of the same pipet or different pipets into the stock bottle can be avoided. The same precaution should be followed with the colloidal gold solution; namely, pour out about 60 ml. of this material from the stock bottle and reject the surplus.

Attention must be called to the fact that the smallest amount of saliva flowing into the pipet and touching the gold solution will inhibit normal precipitation of the colloidal gold solution. It has been found, too, that the soluble sizing from toweling used to dry a beaker has pronounced protective powers.

In order to check the gold solution and the salt solution, the addition of 0.4 ml. 1% salt solution to 5 ml. gold solution should produce no precipitation after standing at room temperature 18 hr., whereas the addition of 1.7 ml. should produce complete precipitation of the gold in the same period of time. The addition of 1 ml. 1% solution of sodium chloride to 5 ml. gold solution should produce, upon standing overnight, a color change adjudged to be midway between a 1 and a 2 reading. The ultimate test, of course, is to use the spinal fluid from a known paretic, which will give the typical first-zone curve. If the gold solution should exhibit evidence of protection, a fresh 0.4% salt solution must be prepared with which the gold solution must again be tested. If the same results are obtained, it is probable that the gold solution has become protected through contamination; if an improved result is obtained, contamination of the original salt solution by a protectant is indicated; on the other hand, if an undue tendency for the gold solution to precipitate is found and an improvement is then noted with the fresh salt solution, the presence of a precipitant in the original salt solution may be inferred.

Mastic test—method of Cutting

The mastic test has been proposed as a substitute for the gold test, but it is not as useful a test in the differentiation of the various types of syphilitic diseases of the nervous system. Complete precipitation of the mastic corresponds to complete decolorization of the colloidal gold reagent, whereas partial precipitation of the mastic corresponds roughly to the purple or blue of the colloidal gold test. The method used by Cutting follows.

Reagents:

1. Mastic solution.

Gum mastic, U.S.P.	0.2 gm.
Absolute alcohol	20 ml.
Freshly distilled water . . .	80 ml.

   (a) Dissolve the gum mastic in the absolute alcohol and centrifuge to clear.
   (b) Pour the filtrate rapidly into the distilled water and mix well.

2. Alkaline saline solution.

Potassium carbonate, reagent grade . .	5 mg. (0.005 gm.)
Sodium chloride . .	1.25 gm.
Distilled water . . .	100 ml.

   (a) Dissolve the salts in the water.

Procedure:

1. Arrange a series of 6 small test tubes. In the first, place 1.5 ml. alkaline-saline solution and in each of the others place 1 ml. To the first tube add 0.5 ml. of the spinal fluid, which must be completely free from blood. Mix by

sucking the fluid up into the pipet and expelling it; then transfer 1 ml. to the second tube. Again mix and transfer 1 ml. to the third tube and continue down to the fifth tube, discarding the 1 ml. portion which is removed from this and leaving the sixth tube with alkaline-saline solution alone to serve as a control.

2. Finally add 1 ml. of the mastic solution to each tube. Mix well and set aside at room temperature for 12-24 hr. or in the incubator for 6-12 hr.

Tubes in which the reaction is complete will show a heavy precipitate with clear supernatant fluid.

Interpretation: As stated before, this reaction is read as one reads the Lange colloidal gold test reaction. If there is no precipitation of the colloidal suspension in any of the tubes, this is a negative reaction. Any precipitation is considered a positive reaction and the characteristics of the precipitation in the various tubes determine the interpretation; e.g., most precipitation in the first tubes is read as paretic zone, precipitation in the center tubes as syphilitic zone, and in the last tubes as meningitic zone.

Quantitive test for protein

See p. 49.

SUGAR ESTIMATION IN SPINAL FLUID

It is generally believed that the sugar values in spinal fluid vary between 50 and 75 mg./100 ml. Estimation of the amount of sugar in spinal fluid is important because a low sugar content is diagnostic of certain conditions. For instance, there is a low sugar content in acute and subacute meningitis due to the glycolytic action of the organisms present. It also has been noted that there is a low sugar content in cerebrospinal fluid containing blood. This is due to the action of glycolytic enzymes which are present in the blood, which serve to reduce the normal sugar content of the fluid. A low sugar content has been seen in cases of so-called aseptic meningeal reaction due to the glycolytic action of cells in vivo. The level of the spinal fluid glucose should always be measured together with a blood sugar specimen obtained immediately prior to the spinal puncture since the procedure in itself may increase the C.S.F. glucose contents. If possible, the patient should be fasting and there should be no delay in performing the test (or in preparing the filtrate).

The lumbar and cisternal fluid sugar amounts to 10-20 mg./100 ml. less than the blood sugar but still reflects blood sugar fluctuations. The ventricular fluid sugar approaches the blood sugar level.

Cerebrospinal fluid sugar:

- Increase:
  - Diabetes
  - Encephalitis (some forms)
- Decrease:
  - Infections
    - Bacteria
    - Fungi
  - Hypoglycemia
  - Metastatic tumor
- Normal:
  - Viral infections
  - Encephalitis (some forms)

For procedure see clinical chemistry, p. 83.

TEST OF SPINAL FLUID FOR SYPHILIS

See discussion on serology.

BACTERIOLOGIC EXAMINATION OF SPINAL FLUID

See discussion on clinical bacteriology.

TRYPANOSOMES IN SPINAL FLUID

See discussion on parasitology.

References for cerebrospinal fluid

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Section Four

FECES

The examination of the feces, while generally stressed by clinical teachers as a very important means of diagnosis in medical and surgical cases, is seldom thoroughly performed. The leading gastroenterologists are undoubtedly examining stool, just as they are giving barium enemas, and making roentgenologic examinations of the canal, but most physicians are not adequately utilizing this type of examination. A routine chemical, bacteriologic, and microscopic examination of the stool of every patient is not indicated, but in certain selected cases, this examination will be of considerable aid. The microscopic examination and the more important chemical tests can be quickly performed. A search for pathogenic amebae is frequently indicated.

Physicians make exhaustive examinations of all parts of the patient except the anus and rectum; in most cases this part of the physical examination is superficial, and consequently much valuable information is overlooked. The indifference to the possibilities of help from routine stool examinations, lack of concise information as to technic and interpretation, and esthetic objection to handling the material, all have conspired to make this field of laboratory work practically a terra incognita to most workers and students.

In southern states one finds more interest and more real work being done on stools than in any other section of the country because of the prevalence of intestinal parasitism in that district and the impossibility of making a definitive diagnosis in any other way. We find a growing interest in stools, too, on the part of pediatricians, but we believe much more emphasis must be placed on this subject.

Chapter 108

Laboratory examination of feces

STANLEY REITMAN

NORMAL FINDINGS

It must be remembered that what constitutes the normal stool is quite a variable factor. There is no normal standard regarding either the frequency or absolute character of the stools. The number, character, weight, consistency, and color of the passages must be accurately ascertained in the individual case before one can draw reasonable and proper deductions.

In order to appraise the individual case, certain fundamentals must not be overlooked. The feces consist of the indigestible and undigested remnants of food, certain constituents such as material secreted by the intestinal wall, and myriads of bacteria that may or may not belong to the normal intestinal flora.

The usual amount of stool is 100-200 gm./day, but this quantity is dependent upon the amount and character of food ingested. Besides the diet, the amount of feces passed depends upon the adaptability of food to the peculiarities of the individual's digestion, the rapidity of intestinal absorption, the amount and character of the digestive juices, such as the bile and pancreatic fluids, the amount of mucus discharged by the intestinal mucosa, and the biologic characteristics of the bacterial flora of the canal. The age of the patient, his habits, whether he leads an active or a sedentary life, his occupation, etc., all influence the number and weight of the stools.

The stools of patients with diarrhea lasting several days show undigested muscle tissue if they have been eating meat, also unchanged connective tissue elements. The predominant ingredient of a stool that is associated with inflammation of the small intestine is mucus, thoroughly mixed with food remnants.

COLLECTION OF THE SPECIMEN

In order to gain the maximum information, the stool specimen must be properly collected and submitted to the examiner as rapidly as possible. This is particularly true with respect to examination for parasites, when, in order to detect their motility, the stool should be examined while still warm. For routine bacteriologic and chemical examinations this is not vital. The best results for all purposes are obtained when conditions are such that preservatives are not needed. For long distance transportation a chemical preservative must be added, and this sacrifices some of the data obtainable from the fresh, unchanged specimen.

Physicians, as well as laymen, are prone to send both sputum and feces specimens in unwieldly, impracticable, and dangerous containers. The Post Office regulations in this country very properly compel one to take precautions against transmitting disease via infectious material in the mails. Specimens may safely be mailed if they are placed in double, tightly closed metal containers. The stool should be collected in a wide-mouthed, chemically clean and sterile bottle. (We recommend the sterilization of all glassware at 170° C. for 2 hr. in the dry heat sterilizer.) The container must be chosen with the following ideas in mind: (1) to keep the material in much the same condition as it was when passed from the patient and (2) to withstand the rough handling to which it may be subjected in

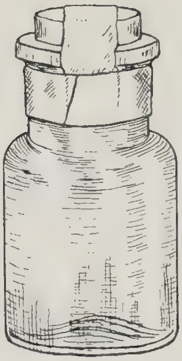

Fig. 693. Taped bottle for collecting specimen of feces.

transit. Despite reasonable care in transit many very severe strains are placed upon such containers; they must be carefully packed so as to withstand the severest of ordeals in shipment.

The receptacle and its packing must be so prepared that it will be neither a menace to the health of those handling it nor offensive to the sensibilities of those who must examine it. Containers leaking fecal material from all sides, as from a Mason jar, for instance, require great care and time on the part of the laboratory worker to prevent infection and contamination of laboratory material. Above all, paper receptacles are to be condemned and never used.

Fill the bottle only about ⅓ full so as to allow ample space between the stopper and the top of the fecal contents to prevent the stopper from being forced out by gas formation. The stopper should be of cork and should fit tightly enough to prevent leakage. It should be long enough to project well above the bottle so as to facilitate removal. It is well to secure the cork with adhesive strips.

The use of 20% glycerin in physiologic saline has been suggested by Cumming, who claims that it has an inhibitory effect on the growth of the colon bacillus and other fecal microorganisms. His solution is made by adding 20 ml. glycerin to 80 ml. 0.9% sodium chloride. To 20 ml. of this solution a mass of feces about the size of a split pea is added. Shake well to make a uniform suspension. Do not use a larger amount of feces since the inhibition of *Escherichia coli* might not be controlled when larger amounts are used. According to Hazeltine[1] this method gives admirable results on specimens artificially contaminated with laboratory cultures of *Salmonella typhosa* and should be applicable in practice.

When the stools are to be examined in a hospital laboratory adjacent to the hospital-domiciled patient, they may be received directly into a clean sterile granite-ware bowl and taken immediately to the laboratory.

Stools submitted for examination for living amebae should be examined while still warm. Patients with dysentery due to pathogenic amebae may show these parasites only during the activity of the process when the patient has frequent bloody or purulent passages. The material should be examined at once, using a warm stage. If the patient cannot come to the laboratory, or the laboratory worker cannot take his microscope and other material to the bedside, then it is best to have the attending physician make half a dozen slides from the fecal matter, allow them to dry, and ship them to the laboratory. An examination of the stained specimen can be made for cysts; this is the most reliable method of diagnosis. In cases in which an examination is being made of the feces for amebae in a fresh state, the bottle or receptacle containing them should be placed in an incubator and kept warm while the technician is adjusting the microscope and warm stage. Refer to Chapter 55 for details relative to examination of stools for intestinal protozoa.

In general, feces should be examined as expeditiously as possible because of the rapid tissue and bacterial changes. The isolation of *Salmonella typhosa,* for instance, increases in difficulty in proportion to the age of the stool. A specimen that has been quickly examined for typhoid bacilli will often show a good culture, whereas the same stool kept for a while and then cultured will show no growth of the organism. Certain changes of decomposition take place in the stool; the overgrowth of one or more hardy microorganisms prevents the

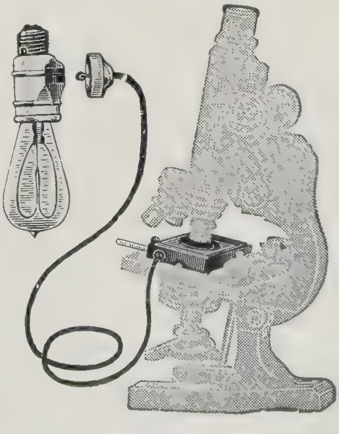

Fig. 694. Warm stage. (Courtesy E. H. Sargent Co., Chicago, Ill.)

growth and recognition of *Salmonella typhosa* in the stools of suspected carriers. The administration of a cathartic increases the number of bacilli in the stools. It is good practice, therefore, to precede the search for typhoid bacilli in suspected carriers by the administration of a saline cathartic. Bacteriologic examination of stools is discussed in detail in Chapter 31.

As Cammidge has pointed out, the feces of infants must generally be scraped off diapers and are therefore contaminated more or less with material derived from dusting powders or ointments which have been applied to neighboring parts. This must be taken into account in the chemical and microscopic examinations of infants' stools. The feces should be free from urine to prevent interference with the reaction, the ammonia content, chloride estimation, etc. With adults it is not difficult to prevent the contamination of stool with urine. In the case of infants the nurse may prevent contamination by placing a large pad of absorbent cotton over the urethral orifice while collecting the stool for the analyst. A stool that is strongly alkaline or that has an ammoniacal odor with a well-marked reaction for chlorides can be instantly proved to be contaminated with urine because the stool never shows more than a bare trace of chlorides. The **presence of urine** can be quickly proved as follows.

Make an aqueous extract of the stool with boiling water. Filter. Acidify with nitric acid and add 10% silver nitrate drop by drop. An uncontaminated stool will show only a slight opalescence due to formation of silver chloride. Stool containing urine will give a heavy white precipitate, indicating a large amount of chloride.

Only a small amount of feces is necessary for the usual routine stool analysis. In most cases in which a quantitative chemical analysis is desired, the stool must be collected for 3 days and the entire specimen submitted. A complete chemical analysis of stools is unnecessary except under special conditions. To determine the metabolic changes in a given case, one might make such a chemical analysis in conjunction with the estimation of the chemical intake of food, the study of the blood chemistry of the individual, and the chemical study of the urinary constituents. As a rule, the stool examination should consist of a determination of certain essential factors, macroscopic and microscopic. Under special conditions this examination may be varied to include a complete bacteriologic study, qualitative and quantitative.

EXAMINATION OF STOOLS

The examination of stools is divided into 5 studies: (1) macroscopic, (2) microscopic, (3) microchemical, (4) chemical, and (5) bacteriologic.

MACROSCOPIC EXAMINATION

The macroscopic identification of parasites may be summarized as follows: the proglottids of the tapeworms, *Ascaris lumbricoides,* and *Enterobius (Oxyuris) vermicularis* are likely to appear spontaneously in the stools. *Ancylostoma duodenale* and *Trichuris trichiura* appear only as a result of definite therapeutic attempts to expel them. Rarely one finds insects and their larvae. Workers must be sure that stools are not contaminated **after** their expulsion either accidentally or purposely by malingerers or psychopathic individuals.

The number and variety of foreign bodies that have been found in stools are legion. They may vary from meat skewers, splinters of wood, eggshells, garden earth (from imperfectly cleaned vegetables), kernels of nuts and fruits, pieces of tobacco leaf, tea leaves, etc. It is remarkable to note that sharp instruments such as needles, pieces of a knife blade, and glass balls may pass the entire length of the canal and may be expelled without injury to the wall. It must not be forgotten that some of these foreign bodies may be placed in the rectum by psychopathic persons.

The macroscopic examination is very important.

Place a portion of the stool on a flat dish. Note the color, consistency, quantity, and mixture with large mucous particles, blood, and undigested food remnants. For closer examination some of the stool should be mixed with water and this diluted material put in a Petri dish with a dark background. The brown particles that will then be noted should be examined microscopically.

Pathologic macroscopic findings are as follows:

1. Undigested food remnants, such as connective tissue, remnants of potatoes and meats, and pieces of fat.
2. Mucus, pus, or blood.
3. Pieces of human tissue.
4. Intestinal stones and gallstones.
5. Worms.

The **physical characteristics** of the stool should be investigated.

Color: Under normal conditions the color of the stools may be light yellow to blackish blue, dependent in a measure upon the character of the food ingested. Exposure to the air and to light causes the stool to become blacker. The fecal color is due to bile pigment plus the presence of foreign pigments of foods and drugs. The ingestion of blackberries or huckleberries causes black stools; chocolate gives them a dull gray color; cocoa makes them light gray. The effect of starchy food is to produce a yellow stool; of chlorophyl from vegetable material, a greenish shade. Fats produce the classical clay-colored stool. Certain drugs, such as bismuth and iron, produce a black stool; senna, rhubarb, santonin, and calomel give a yellowish green cast.

The normal color of the fecal material is due to the presence of urobilin and stercobilin derived from the bilirubin of bile; the intestinal bacteria convert bilirubin into the urobilins. Neither of the bile pigments, bilirubin nor biliverdin, is ever normally encountered in a stool unless the bile is passed unchanged down the intestinal tube by unduly rapid peristalsis. The darkening of the stool after exposure to the air is probably due to the conversion by bacteria of urobilinogen into the urobilins. The color of stools is mainly dependent upon the character of the diet. If one is to be influenced by color of the stool in estimating a fault in health, then he must inquire very carefully as to the diet taken, whether mixed, whether it contains vegetables or fruit, etc. An inquiry of this kind will usually explain the color.

Blood: Blood is not easy to identify visually in the stool because the bright red color is lost after the blood remains some time in the intestine, changing in color to the brown of hematin. A large quantity of blood gives the appearance of cooked tea. If blood comes from the lower bowel, it will usually appear on the surface of the stool and can be detected. It is always red if it comes from the rectum and is darker if it comes from a higher point. When summarizing the evidence presented by either the macroscopic or the chemical analysis for blood in stools, one must know whether there are any hemorrhoidal changes present, also whether there is a rectal fissure, as well as polyps, rectal adenomas, carcinoma, or ulcer. Attention should be called to the gross appearance of stools contaminated with large quantities of blood coming from points higher up, notably the tarry color due to a bleeding duodenal ulcer, typhoid stools with bleeding, or in severe dysenteric conditions.

Blood mixed with pus is seen in dysentery. Mucus and blood are seen in mixed cases of intussusception and polyposis intestinalis. Blood is found, too, in cases of embolism of the mesenteric arteries.

Form: The form of the fecal material gives an idea of certain conditions. The stools in prolonged constipation are hard and often ball-shaped, sometimes grooved by the intestinal bands. There is a characteristic scybala formation called "goat droppings" seen in cases of spastic constipation, or one may see pipestem or ribbon-shaped stools. In general, it may be said that the formation of scybala, due to small bulk of feces and lack of moisture, is evidence of spastic constipation.

Liquid and semisolid stools are indicative of irritation in the intestinal tract, and the passage of this type of stools is termed diarrhea. There are varieties of these liquid formations, sometimes spoken of as characteristic of certain definite disease states, such as the "pea-soup" stools of early typhoid and the brilliant red stool of the later period. In cholera the so-called "rice-water" stools are seen.

Odor: The odor of feces is due to the presence of indole and skatole which are formed as a result of the putrefactive processes that normally occur in the intestine. These putrefactive processes result from the action of bacteria upon protein present in intestinal contents. The most important products formed are indole, skatole, paracresol, phenol, *p*-oxyphenyl-propionic acid, *p*-oxyphenylacetic acid, volatile fatty acid, hydrogen sulfide, methane, methylmercaptan, carbon dioxide, proteoses, peptones, peptides, ammonia, and amino acids. Of these products indole and skatole in the feces give the disagreeable odor, whereas indole, skatole, phenol, and paracresol appear in the urine as ethereal sulfuric acids. Methane, methylmercaptan, and hydrogen sulfide may add to the disagreeable odor produced by indole and skatole. The more meat digested by the patient, the more the odor; it is less marked in stools from a vegetable diet and hardly noticeable in stools from a milk diet. This explains the fact that stools of infants on milk diets are practically odorless except under pathologic conditions.

Casts: Casts of the bowels are occasionally found. They may be passed in shreds or in the form of a complete cast of the intestine. This condition has been designated by Nothnagel as enteritis membranacea or colica mucosa. They may be a foot or more long and may be passed, accompanied by severe tenesmus, without any fecal material. These cases are seen more commonly in women than in men. They are found, according to Greene,[2] associated with 2 conditions aside from the causes mentioned, namely, impairment of general nutrition and spastic constipation. The nature of these casts may be determined by the **Pariser method:**

Treat them with sublimate alcohol and then with Ehrlich triacid stain; they turn green if mucoid, whereas they turn red if fibrinous. Details of the test are given below.

Undigested food particles: These particles may be seen and identified by the naked eye. Pour liquid stools into Petri dishes. Thick pasty stools are best stirred with water and then poured over a fine-shred sieve. The sieve described by Einhorn is very useful to detect concretions. Another method without the use of a special sieve consists of tying a double thickness of cheesecloth over a ring of wood or on to the edge of a pail. Kemp has described a method that is simple and practical. A circular wire is fitted to the toilet seat and to this is attached a bag made of double thickness of cheesecloth which dips into the pail. The patient defecates into the bag. Water is poured through until only the more solid parts remain. These are then scraped off and examined. The same thing may be accomplished by means of a colander over which is laid a fine meshed wire screening.

Mucus: Mucus that appears in the stool is usually visible macroscopically. In very small quantity it is difficult to find, but in larger quantity it can readily be seen and grasped with forceps. Cases in which it is found only with the aid of a microscope are rare, but they do exist. Our interest therefore is in mucus that can be found macroscopically.

Tests for mucus may be made as follows: Wash a few particles of the supposed mucus in water and stir in a test tube with 2½% mercuric chloride in alcohol, using a glass rod. The larger particles are thrown down by the hardening effect of the mercuric chloride in alcohol. Sediment the specimen, add distilled water with a few drops of Ehrlich triacid stain, sediment again, wash in water, and examine microscopically. The mucus particles, provided they are not too rich in cells or fat, are stained bluish green or green; all the animal cells are stained red.

Mucus is precipitated with acetic acid. The precipitate is not redissolved by an excess of this acid. Mineral acids added slowly cause a precipitate; added rapidly they dissolve the mucus. Alkalies cause it to swell and dissolve. Alcohol causes shrinkage. Thionine stains mucus violet; other material remains blue. In order best to obtain this reaction, take care that there is not too much mixture with other material and that the reaction of the specimen is neutral. Other aniline dyes stain only the cells present. Iodine stains mucus a diffuse yellow color. The **Hecht method**[3] is a mixture of brilliant green (2%) with neutral red (1%). Stain a few minutes. This gives a light red color to mucus, which is in sharp contrast to the rest of the field, which is green. Nuclei and cell membranes appear reddish violet.

The amount of mucus present in stools may range from very small particles to enormous masses.

The mucus is usually present in clumps or small masses which, in water, appear to have irregular edges. At times there are the so-called boiled sago nodules, the composition of which has been much in dispute. The consistency of mucus masses varies from extreme softness to leatherlike hardness. Pure mucus masses are usually soft, the harder particles consisting of other material as well as mucus, namely, albumin, fat, and many cells. Mucus is transparent, but when contaminated with other substances, such as fat or albuminous material, it becomes cloudy. It imitates the color of the fecal mass in which it is found, varying from the brown color of hydrobilirubin to the orange red and green color of bilirubin and biliverdin; blood elements, of course, make mucus reddish or red brown.

Washing the stool gently with water makes it comparatively easy to find the smaller particles. Mucus appears under the microscope as structureless material, more or less transparent, in which may be embedded epithelia, pus cells, red blood cells, bacteria, protozoa, air bubbles, detritus, food remnants, and crystals. Mucus from the upper tract is more likely to be entwined with food residue than when it originates from a lower point. The bacterial contents vary, being very scant from cases of mucous colitis. Note 2 points, the irregular lines that traverse it and the irregularity of its boundaries.

Only visible mucus is found under pathologic conditions. The exceptions to this are the meconium and the stools of young infants.

The most important consideration in the study of mucus is its **source.** Usually, mucus comes from the large intestine. The small intestine does not produce mucus with the same facility as does the large bowel. If mucus is found in diarrheal stools very finely subdivided, as in typhoid fever, tuberculosis, and cholera, it may be assumed that it comes from the small intestine. Further proof of the small intestine as the source of the mucus is the impregnation of the mucus with unchanged bilirubin.

The manner in which mucus is mixed with stools may indicate the source; if the well-formed stool is covered with mucus or if the mucus dips down between the scybala, it is coming from the lower end of the large intestine. The ribbonlike masses that are called membranous colitis mucus come from the sigmoid flexure of the colon. If mucus is intimately mixed with a "soupy" stool, the material comes from the upper part of the large intestine; in fact, the smaller the particles and the more intimately they are mixed, the higher their source.

The pathologic process underlying the undue pouring out of mucus into the intestinal canal is usually a catarrhal process, with increase in the glandular activity and a diffuse inflammatory change in the entire mucosa.

The degree of inflammation may in a measure be determined from the transparency of the particles of the mucus. Our conception of a very severe attack is a mucus that is very cloudy, due to the content of epithelial and pus cells. There may, however, be a catarrhal inflammation without mucus in the stool, so that absence of mucus does not exclude inflammation. The appearance of the sagolike clumps of mucus occurs in the stools of children as an evidence of severe mucous inflammation. Heubner, in explaining this formation, thinks that the nodules of mucus are formed from the follicles in the intestinal wall, whereas Kelsch believes they are pieces that are squeezed out of the Lieberkühn follicles. The presence of a large amount of pus with mucus indicates ulcerative inflammation; with blood, a diagnosis of ulcerative enteritis (dysentery).

Fibrin: It is difficult to identify fibrin in the stools either visually or even chemically. Autopsy findings of fibrin are common in dysenteric patients, but there it is attached to the walls of the intestine. It is logical to assume that it is cast off and appears in the stool, but it cannot be proved by an examination of the stools. Fibrin masses are found especially in membranous colitis.

Tissue remnants: Tissue remnants emanate either from tumors of the intestinal wall or from necrotic mucous membrane changes. In the first group we note polyps, pieces of hemorrhoidal nodes, carcinoma, and occasionally lipoma. Necrotic pieces are seen in ulcerative processes, such as invagination cases, typical dysentery, twisted off pieces of adenomatous polyps, or pieces of ulcerated neoplasms.

Very small necrotic masses have been seen in the stools of patients with typhoid fever in the second or third week.

The greatest stumbling block to an attainment of a good working knowledge of the material commonly seen in stools is the presence of nonpathologic substances or artifacts. These are fly larvae, unusual pieces of food, pieces of goods, pieces of apparatus, and other objects that may come in contact with the stools or containers. This must constantly be borne in mind; in fact, a microscopic study of all such foreign matter is very good preliminary practice in preparing oneself for coprologic expertness.

Other organic material: Other organic material may be parasites or various calculi. Among **calculi** are gallstones which are identified by their smooth and facetted surfaces and their light weight. Chemical means will fully identify them. Although all sizes of gallstones may be found, the 2 extremes, very large and very small stones, are rare: the very large stones lead to obstruction and are not passed; the small stones are so easily broken up that they are no longer recognizable when they reach the lower end of the canal. The rare pancreatic stones have no known characteristics to make their identification with the naked eye easy. According to Fleiner of Heidelberg, their surfaces are facetted and rough.

MICROSCOPIC EXAMINATION

The microscopic examination of the stool may be made either a very simple procedure or a very complicated one. It depends upon how far we wish to go and upon the object of our investigation. We may simply wish to survey the crystals present, or the food remnants, or to verify our naked eye judgment or to prove the presence or absence of eggs of the more common parasites such as the hookworm or the tapeworm; we may wish to make a simple smear for tubercle bacilli or to make a smear for other microorganisms; in short, our microscopic examination may be directed to the solution of one problem or a great many. We usually are given a clue from the clinician as to what he particularly wants investigated.

Preparation of specimen

In general, the following material is needed for a microscopic examination of stools: glass slides and cover glasses; teasing needles; a good centrifuge; test tubes and small funnels, pieces of sterile gauze for filtration; sterile Petri dishes; and culture media for identification of the more common varieties of bacteria such as the colon and typhoid bacilli.

It is inadvisable to add any chemical substance to the stool to reduce the odor since these substances always cause a distortion of the picture and render the examination of doubtful value.

The simplest examination consists in taking a small piece of the stool on a toothpick and rubbing it on a glass slide, covering with cover glass and examining. Portions from various places should be taken, from the solid parts, and from softer and more liquid parts. Be sure the specimen is as fresh as possible to eliminate the question of alterations from standing. If the stool is not particularly fresh, take the specimen from the inside of the masses rather than from the outside. In exerting pressure on the cover glass elastic tissue and watery material will not remain pressed out, mucus and fat containing stools remain pressed out. If a grittiness as of glass against glass is noted, crsytals or sand may be present. Thin watery stools may be sedimented and thicker heavy stools may be slightly diluted with water or saline in making this examination. The mixture may be made on the slide. Examine with the low- and high-power dry lenses. To isolate certain parts of the stool, wash repeatedly, shake, sediment, and pour off the supernatant fluid. Remove the large particles which are visible to the naked eye and examine the sediment.

Sedimentation can best be accomplished with the use of a centrifuge tube very much like that devised by Hopkins for the counting of bacteria. Run the centrifuge at moderate speed. Note a sharp line of separation between the sedimented portion and the supernatant fluid. In the cloudy portion just above the sediment the various bacteria and the fatty acid needles are found in fatty stools. Note foamy fat on the surface in cases with much fat in the stools. In this upper portion are seen small particles of vegetable material, mucus with air bubbles, and particles of connective tissue cells. In the deeper portions of the sediment are crystals of large size such as triple phosphates, and the hair-like covering of vegetables, and the large muscle remnants.

There are a number of other more elaborate methods of washing and sedimenting the stool for microscopic study, but the above method will generally suffice.

Microscopic findings

Epithelia: Epithelial cells from all portions of the tract are likely to find their way into the stool. The character of the cell points to the origin, but pure histologic pictures as they occur in the mucosa do not exist in the stool because the digestive juices have a powerful effect in changing the morphology. There may be pure unchanged stratified squamous epithelial cells from the region of the anus, but even this is rare. They appear, however, less changed than cells from points higher up; for instance, the cylindrical cells from the small and large intestines. These squamous cells closely resemble the cells that fall into saliva from the mucosa of the mouth. They are very large nucleated cells and are not to be confused with the nuclear-free cells of meconium and infant stools which have originated from the vernix caseosa. Pure types of squamous cells are rubbed off, unchanged, from the anal orifice by passage of the stool. Cylindrical cells from points higher up are seen as slightly distorted cells, found in washings of the gut or in thin soupy stools of typhoid, diarrheas of children, tuberculous enteritis, and cholera. They have clear nuclei and granular cytoplasm. They may appear singly or grouped as they are in the intestinal wall, arranged side by side. These cells are sometimes swollen with fat droplets. One may see the cells described by Nothnagel as "vanishing" or epithelial cell "ghosts" bound up with mucus particles. They

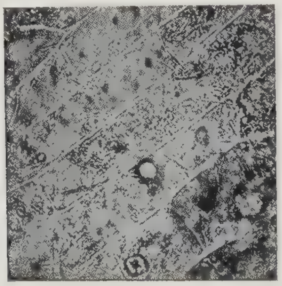

Fig. 695. Vegetable cells in feces.

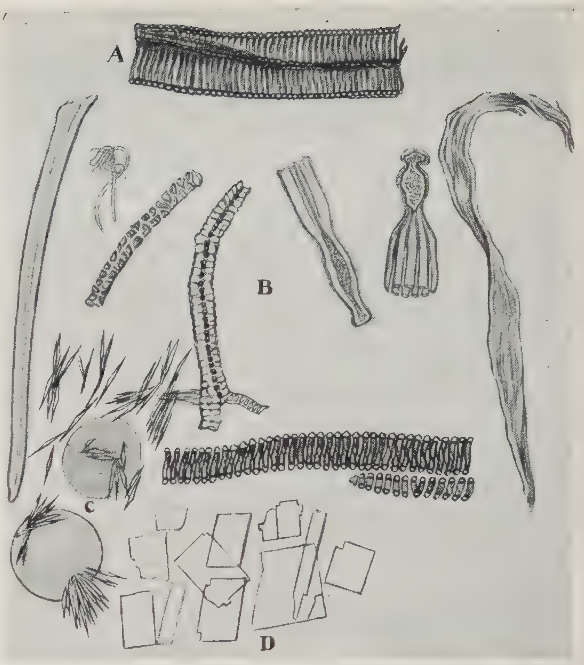

Fig. 696. Microscopic findings in feces. **A** and **B,** Vegetable fiber remnants, banana (**A**) commonly mistaken for tapeworm section; **C,** fatty acid crystals; **D,** cholesterol crystals.

are supposedly degenerated cells according to Leube, or amyloid cells according to Lambl. They are small and homogeneous and have no nuclei. They are refractive and apparently have lost their granules. They are frequently spindle shaped. Nothnagel believes they are cells that are changed by abstraction of water. Kitagawa, however, stated that he found these cells in watery stools in diarrhea and that they are the result of a degenerative process, either hyaline or coagulation necrosis. Epithelial cells in the feces are usually not stained. Rarely are they yellow colored from bile. Nothnagel believed that the staining with bile is an intervital process and does not occur post mortem.

A sharpness in outline of the cytoplasm of the epithelial cells may be produced in the usual way by the addition of acetic acid. Osmic acid will stain fat granules black.

The diagnostic significance of epithelial cells in abundance is their indication of the existence of catarrhal processes. The occurrence of a large amount of mucus and a paucity of cells evidently shows that the derangement is more a result of a nervous irritability of the intestine than a true inflammatory process. This is especially true in cases of enteritis membranacea. Toxic disturbances of an intense nature, as in cholera, cause a great exfoliation of cells. One cannot conclude simply because the epithelial cells are bile stained that the disturbance is in the small intestine. Other signs must be at hand to make this diagnosis. The presence of the "vanishing ghost" cells does not prove their source the large intestine; when mucus is present as well, this is probable. The presence of half-digested cells with mucus indicates a small intestine disturbance, especially if the cells are bile stained. This picture is seen in typhoid fever and tuberculous enteritis, in which pus cells are also present.

Leukocytes: Leukocytes are not commonly found in feces. They are abundant naturally where there is pus formation, as in ulcerative conditions in the tract. One may have difficulty in identifying them under the microscope, but they may present a perfect contour, with granular cytoplasm and single or multiple nuclei. Eosinophilic granules may be seen; particularly is this true when Charcot-Leyden crystals are found in cases of parasitism. Leukocytes that are large enough to be designated giant cells have been seen in feces. They are much larger than the flat cells found in the mucosa of the mouth. They show vacuoles. They are seen in tuberculous enteritis. Schmidt noted phagocytes in a case of dysentery. The shadows of leukocytes are sometimes seen. Small, broken down leukocytes are frequently seen enmeshed in mucous particles. The presence of a few leukocytes in conjunction with epithelial cells in infantile stools indicates an irritation process.

Pus: Macroscopically the grayish masses which microscopically prove to be collections of pus cells may be observed. If yellow material which is unmistakably pus is present, one must think of para-intestinal pus pockets in the region of the large intestines. Pus coming from a higher point in the small intestine would undergo changes before being expelled with the stool mass so that it would not be readily recognized macroscopically. In the various ulcerative processes of the large intestine and even of the small intestine, such as dysentery, colitis ulcerosa, syphilis, neoplasms, tuberculosis, and typhoid, pus may be intimately mixed with the stool and is difficult to recognize as such.

Pus cells do not appear in feces in abundance in catarrhal states, as is the case in sputum of catarrhal inflammation of the respiratory tract. The presence of much pus always indicates an ulcerative pathology with but one exception, namely, colitis with severe inflammatory changes, with pus, blood, and mucus in the stools, without ulceration. One must remember that there are certain types of ulceration without pus, e.g., the perforation of typhoid ulcers.

Erythrocytes: Blood must be very fresh in order that the cells be seen intact under the microscope. Even then the crenated or crab-apple form are usually seen because of the acid reaction of the stool. Only a large number, as expected in an ulcerative or intensely inflammatory condition, will yield shadow forms which show no color. The finding of red cells in feces usually indicates ulceration. Exceptionally they are found in venous stagnation. Well-preserved corpuscles are seen in cases of hemorrhage from stasis low down (hemorrhoidal vein bleeding).

Detritus: Besides the various elements already described, there are found in stools microscopic particles that are extremely small and impossible to identify. This material is called detritus. It is intimately digested muscle remnants, plant cells, bacteria, epithelia, and isolated nuclei too far changed by the digestive juices to be recognizable. The presence of a large quantity of detritus is a good indication of the chemical and mechanical efficiency of gastrointestinal digestion.

Crystals: Many crystals are found in feces, some derived from food, others from chemical changes in the intestine.

Triple phosphate or ammonium magnesium phosphate crystals are the commonest. They vary in size, some so small that they can be covered by calcium oxalate crystals, others very large. The best developed crystals are seen in fluid stools. Their shape is that of the "knife rest" or "coffin lid," but some are more or less irregular (Figs. 635 and 636). Some are so large that they cover 2 microscopic fields, in which case they can be seen in the specimen with the naked eye, as a small white point on the slide. They sometimes appear as 3- or 4-cornered splinters representing breaking down of the "coffin-lid" formation. Triple phosphates are never bile stained. They are freely soluble in dilute acetic and other acids. They are insoluble in ammonium carbonate solution which distinguishes them from neutral phosphate crystals which they closely resemble. Some claim that they are found in normal and abnormal stools. Szydlowski found them absent in but one condition constantly, i.e., icterus. Lynch states that they are never found in meconium. When found, we must be sure that there is no urine contamination.

Di-calcium phosphate or neutral phosphate of lime crystals appear in the form and size of those found in urine, larger or smaller sheaves with the points lying together. They may occur as rosettes. They are occasionally bile stained, whereas triple phosphates are never bile stained. A homogeneous transparent variety is seen in milk stools, this form being a combination with fatty acids. They are freely soluble in acids and in ammonia, as well as in ammonium carbonate solution. They are found in normal stools, particularly when alkaline stools have been allowed to stand for some time. They have no diagnostic importance.

Neutral magnesium phosphate or trimagnesium phosphate crystals are found in stools showing ammoniacal decomposition. They occur as irregular sheaves, never as in urine as light refracting long rhombic plates. They are soluble in acetic acid and become opaque when ammonium carbonate solution is added.

Calcium carbonate crystals appear as round or dumbbell-shaped crystals or amorphous granules. The addition of acetic or other mineral acids dissolves them with gas formation. Sulfuric acid causes them to assume a sheaflike form. These crystals are commonly seen in the stools of milk-fed children.

Calcium oxalate crystals are found in ingested vegetables that contain them, such as rhubarb, tomatoes, spinach, etc. They are not seen on a milk or meat diet. With a **large** amount of vegetables a moderate number have no significance, but with a **small** quantity of vegetables an enormous quantity of calcium oxalate crystals indicates an intestinal catarrh, showing that cellulose disintegration has been retarded, with an escape of the crystals from the plant cells. In hyperchlorhydria an abundance of vegetables will not result in the appearance of many of these crystals, for these are dissolved by the excess acid in the stomach; consequently there may be an increase of these crystals in the urine. They appear in various forms in feces, as envelope shape, as typical rosettes, and as rhomboid and acicular varieties (Fig. 637). They are insoluble in acetic acid and alkalies but are soluble in mineral acids.

Crystals from medicaments are sometimes found. Dark bismuth crystals are found after ingestion of bismuth subnitrate. The use of salicylate of bismuth gives peculiar crystals of this drug; iron, charcoal, and manganese pass as black amorphous granules.

Cholesterol may at times appear as flat superimposed plates, often with re-entrant angles, but as they rarely show typical forms they must be identified chemically. They are easily soluble in hot alcohol, ether, and chloroform. They are insoluble in water, alkalies, and acids. On adding iodine followed by sulfuric acid there is a play of colors, yellow, yellow red, carmine red, violet, green, blue, with melting of the crystals from the edges. Cholesterol appears particularly in children where there is a large amount of mucus passed. It is commonly seen in meconium.

Charcot-Leyden crystals appear as they do in sputum, as colorless octahedra with sharp margins and generally broken angles. They are commonly seen embedded in mucus. Leichterstern spoke of them as especially characteristic of helminthiasis. His pupil, Buckler, further emphasized this relationship together with the presence of eosinophilia in the blood, but other authorities disputed this as constant or as sufficiently noteworthy to be of value in diagnosis. Charcot-Leyden crystals are found in abundance, it is true, in cases of helminthiasis, but they are absent in other cases. They have also been found in abundance in other conditions, e.g., in the mucus of enteritis membranacea, by Akerlund and Schmidt. They are no more characteristic of any disease when found in feces than when found in sputum, even though they are present in abundance in cases of bronchial asthma.

Hemin crystals are not seen in feces except after ingestion of blood sausage, but **hematoidin** crystals are common. They occur as reddish yellow rhombic plates, columns, needles, or bundles of needles, at times as amorphous masses. Uffelman reported them in infant feces, and Lynch reported them in meconium. Von Jaksch found them in intestinal stagnation hemorrhages or free hemorrhages in adults. They are soluble in ammonia, the solution turning yellow. They are not soluble in potassium or sodium hydroxide solutions. Nitric acid turns them blue.

Bilirubin appears like hematoidin as rhombic needle-shaped crystals or golden yellow kernels. It is found more frequently in infantile stools than in adults.

Uric acid and uric acid salts are not found except as a result of contamination with urine.

Intestinal parasites: Parasites are living organisms which take up residence in another living organism. They may be of plant or animal origin, i.e., phytoparasites or zooparasites. We are here more especially interested in those parasites which take up their residence in the intestinal canal or structures adjacent thereto and that may be detected by systematic stool examinations. Parasites might be occasional or permanent.

Parasitism is relatively common in this country. It is much more common in tropical countries because of certain customs, failure to cook food thoroughly, and ingestion of many foods in a raw state. With the advent of rapid transport by air, many cases are directly imported into this country and are direct centers for infection of other persons. The spread of World War II into tropical lands resulted in many new cases among our military forces who were sent to these places for duty.

A full description of intestinal parasites may be found in the section on medical parasitology.

MICROCHEMICAL REACTIONS

Microchemical reactions are elicited by simply allowing 1-2 drops of a reagent to run in by capillary attraction between a cover glass and a slide after having made a smear of the feces. If the fecal mass is not fluid, it is impossible to do this, in which case drop the reagent directly on the preparation before placing the cover glass in position.

The following reagents are necessary:

1. Saline solution, 0.6%.
2. Glycerin.
3. Alcohol, ether, and chloroform.
4. Acetic acid, 30%.
5. Hydrochloric acid, 3-5%.
6. Concentrated sulfuric acid.
7. Potassium hydroxide, 15%.
8. Millon reagent (p. 1809).
9. Modified Lugol solution
    - Iodine . . . . 1 gm.
    - Potassium iodide . . . . . 2 gm.
    - Distilled water . . . . 50 ml.
10. Osmic acid, 1%.
11. Sudan III (Herxheimer). Equal parts of 70% alcohol and acetone saturated with Sudan III.
12. Glacial acetic acid . . . . . 90 ml.
    - Alcohol, 95% . . . . 10 ml.
    - Pinch of Sudan III.
13. Weak watery solutions of eosin, methylene blue, and safranin.
14. Ziehl-Neelsen carbolfuchsin.
15. Concentrated aqueous solution of Nile blue.
16. Friediger polychrome solution:
    - Saturated alcoholic solution of dimethylaminoazobenzol . 2 ml.
    - Absolute alcohol . . . . 2 ml.
    - Eosin, 0.5% alcoholic . . . . 20 drops
    - Mucicarmine, saturated aqueous solution . . . . . 20 drops

Preparation of specimens

Although it is not necessary to use all these reagents in all cases, one may adopt as a routine the following microchemical procedure in examination of stools.

Make 3 preparations, 1 of the plain diluted feces, 1 mixed with acetic acid, and 1 treated with iodine solution. The first gives a good general picture. In the second the striation of any muscle fibers is rendered more distinct; connective tissue is swollen in contrast to elastic tissue which is unchanged; mucus is coagulated and rendered more distinct; cells are cleared and their nuclei appear sharper; albuminous granules are dissolved; some crystals, such as triple phosphate, disappear, whereas others, such as oxalates, are not affected. In the third preparation undigested starch granules are colored deep blue; partially digested ones are brown or red.

Findings and confirmatory tests

Most of the material found in feces may be identified microscopically. However, there are certain very minute ingredients representing the breakdown of food remnants, called detritus, which cannot be identified.

Meat fibers: It is natural to find a certain amount of fibrous residue in the stool. Outside their mere presence, the quantity on certain diets is of great importance in drawing a conclusion as to the status of intestinal digestion. It is difficult to differentiate meat fibers from flesh, fowl, or fish; the coincident finding of scales of fish would suggest the fibers are of fish origin, whereas the presence of small pieces of feather would suggest fowl origin. The manner of cooking meat plays a part in the finding of meat fibers: well-roasted beef is more likely to remain in the stool than is raw meat. Muscle remnants appear as isolated polygonal pieces devoid of sarcolemma. At times they lie together as if bound by the skeleton sarcolemma. They may be stained yellow from bile and thus attract one's attention immediately on looking through the microscope. Long and cross-cut striations are easily identified in the fibers. The size of these fibers varies from minute forms to pieces that entirely fill the field, under which latter circumstances they can be seen with the naked eye. The longer pieces have parallel sides with cross striations.

Better digested pieces do not show these sharp contours. The cross striations may be seen with the medium-power dry lens. Schmidt states that cross striations in the fibers from fish disappear more rapidly than do those from mammalian flesh. The color of fibers is dependent upon admixture of bile, the usual color being yellow or yellowish green. Nuclei can never be detected unless there is an absolute disappearance of pancreatic juice, in which case there is macroscopic evidence of unchanged meat in the stool.

Microchemical tests: It is difficult to identify meat fibers by microchemical methods because of the admixture of bile elements and the presence of certain unknown factors. One may attempt to remove bile elements by the use of alcohol; bilirubin-stained meat may be freed of this interfering factor with chloroform. Acetic acid swells the fibers and brings out the cross striations fairly well. The addition of potassium hydroxide causes the fibers to dissolve. If a little copper sulfate is added to the potassium hydroxide and the picture watched under the microscope, a slight violet color (the biuret reaction) may be observed. The addition of concentrated nitric acid with warming of the specimen gives a yellow color of xanthoprotein. Millon reagent causes a disappearance of structure, with a red coloration. Picric acid and iodine-potassium iodide solution gives a yellow color. Eosin solution causes the appearance of a red color in meat fibers more quickly than in other parts of the stool.

There are a number of very elaborate methods for exactly determining the amount of meat fibers present in any given stool, together with an estimation of the meat ingested after selected test meals. These methods are of doubtful usefulness. Certain conclusions may be drawn, however, from the presence of a large number of meat fibers in the stool. The process of digestion of meat occurs mainly in the small intestine, due to action of the pancreatic juices. No further digestion of meat occurs beyond the ileocecal valve. Hence the appearance of unchanged or slightly changed fibers in the stool is an indication not of large but of small intestine dysfunction. The disturbance in digestion in the small intestine may be motor or secretory. Unduly rapid peristalsis may be based upon a secretory disturbance in the pancreas and the intestinal glandular apparatus. In some cases, whether catarrhal, ulcerative, or purely neurotic in origin, all factors may be deranged.

Connective tissue: It is very easy to identify connective tissue in the stools with the naked eye, but it is still easier with the microscope. The connective tissue fibers are not found so constantly in feces as are muscle fibers. The finding of connective tissue fibers depends upon the manner of cooking.

Protein: There are casein clots found under pathologic conditions in stools to which Nothnagel gave the name of "yellow mucus" bodies. They consist of casein clumps which resemble short pieces of muscle fibers; but they are not muscular in origin, for they are found 3-4 wk. after the patient ceases to take meat, as in typhoid convalescent states when no meat has been taken for weeks. They appear as minute yellow round bodies; under the cover glass they give an impression of sandy particles. Microscopically they appear as yellow particles or cells lying close together. There is no differentiation of structure with higher power. They contain no formed elements whatsoever.

They are dissolved by the addition of a mixture of warm water and potassium hydroxide. Acetic acid causes partial precipitation of the clump which is insoluble in an excess of this acid. The addition of potassium ferrocyanide solution causes a white precipitate; Millon reagent stains them red. They are not found in normal stools. Their presence, according to Nothnagel and others, indicates a disturbance in small intestine activity, usually an inflammatory condition.

Pieces of ingested egg may at times be seen in the stool as small particles of the skin or coagulated material (fried egg remnants). Meconium "corpuscles," so called, are found under the high-power magnification. They appear as weakly stained, greenish yellow, homogeneous, round or oval bodies lying near epithelia and cholesterol crystals. Schmidt believed they are shrunken epithelia of vernix caseosa.

Fat: Fat substances are frequently found in the stool as neutral fat, free fatty acid, or soaps.

(1) **Neutral fat:** Neutral fat appears in droplets or in scales of irregular form. The fat scales are usually unstained. The fat droplets appear round. Fat droplets are often stained yellow. In stools of infants fat appears just as it does in a microscopic picture of milk.

Microchemical reactions: All neutral fats when heated melt and run together; when cooled, they resume their individual shapes. Fats are insoluble in water, weakly soluble in cold alcohol, soluble in hot alcohol, ether, and chloroform. They are unchanged by the addition of potassium hydroxide. Osmic acid stains fat black but not necessarily all neutral fats: olein and oleic acid are stained black; palmitin, stearin, and their corresponding acids are not stained except with the addition of alcohol. Fat in the animal body contains all these components. Therefore, osmic acid stains fat black but with varying shades dependent upon the proportions of the ingredients just set forth. Substances other than fat may be stained black by osmic acid. The addition of a few drops of Sudan III stains fat red. The Friediger reagent stains fat yellow. Concentrated Nile blue reagent produces a rose red color.

(2) **Fatty acids:** Fatty acids appear as drops or scales and are difficult to distinguish from neutral fat, at times showing characteristic crystals. The crystals appear as fine needles with pointed ends, similar forms being seen in sputum. They also appear as lancet-shaped plates, occasionally seen in infantile stools or as needles that resemble soaps.

Microchemical reaction: They differ from neutral fats in that they are soluble in potassium hydroxide and cold alcohol. They are not bile stained in the stool nor do they take the stains. They are, however, colored by osmic acid and Sudan III like the neutral fats. They stain intensively with dilute carbolfuchsin whereas neutral fats remain unstained. This is the best differential stain.

(3) **Soaps:** Soaps appear as scales or crystals. They show less luster than neutral fats or fatty acids. They may be unstained or stained slightly yellow by urobilin or bilirubin. They usually appear as unstained needles. One variety is round with fine radial striations.

Microchemical reactions: The basis of these soaps is alkali. They are soluble in hot water and alcohol. They are mainly calcium salt combination. Upon heating the preparation they do not melt as do neutral fats and fatty acids. They are not stained by osmic acid or Sudan III.

(4) **Significance of fat in stools:** Infantile stools show a great deal of fat, as neutral fats, fatty acids, and soaps. Adult stools show fat but usually only soaps as scales. They are not found in healthy persons after a test meal. Naked eye appearance of fat means an undue quantity. This fat can be seen as grayish, shining particles of a smeary consistency. Fatty acid crystals are never found in normal stools, but, according to Muller, they are always seen in the stools of starving people. Fat as a pathologic entity is seen in stools in connection with disturbances of the biliary function, affections of the neighboring mucosa, or the mesenteric lymph nodes, where there is increased peristalsis without anatomic changes in the mucosa. Gastric and large intestine disease does not seem to have much influence on the undue appearance of fat in stools. Pancreatic disease does not necessarily show fat in the stool.

Fatty stools are sometimes seen in breast-fed infants, a mass of fat droplets with increased fatty acids and soap needles. Biedert called this fatty diarrhea. The addition of acetic acid and heat causes the entire microscopic picture to appear as fat droplets. The appearance of visible neutral fat in the stools of adults, of course, is pathologic except where castor oil has been taken.

Starch: Unchanged or slightly changed starch granules with concentric or eccentric markings seldom appear in the stools of adults. They are never stained by bile pigments; they are of varying size; and they are usually changed by the process of digestion so that, except in stools of infants, one does not see starch that has not been subjected to chemical or physical agents. The microscope reveals bodies that are of different size from the original starch granule as it was ingested, granules that fail to show the classical markings. Even the ingestion of raw potato gives a starch residue that is not classical, with a corrosion or unevenness of the periphery.

Microchemical reactions: Cold water has no effect on starch granules, but warm water produces a slow swelling. Acetic acid has no effect. Iodine produces the well-known blue color on unchanged starch granules. Lugol solution is recommended for this test (described on p. 467). Rub the solution well into the fecal mass. The cellulose fiber present often hinders penetration of the iodine solution and prevents a reaction. Semidigested starch gives a mahogany red color, indicating it has changed to erythrodextrin. In the final stage of digestion starch gives no color at all with iodine. Certain spores of fungi give a blue color with iodine. As stated above, unlike muscle fibers, starch is never stained by bile pigments.

As starch is present in some of the undigested vegetable remnants, care must be taken in the preparation of specimens to prevent these fibers from giving up their starch granules with consequent false interpretations. The presence of starch granules intact in the stools of adults points very definitely to derangement of the activity of the small intestine; hence one must be sure that the granules seen are eliminated as such and are not pressed out of vegetable residue. Starch in vegetable residue is found after the ingestion of peas and beans, especially in dried forms. Pancreatic insufficiency alone does not cause starch granules to appear in the stool. The activity of the intestinal bacteria is responsible for this fact; they evidently digest the starch when the pancreatic juices fail to do so. When, however, there is increased peristalsis with this insufficiency, starch is found in the stool mass. The appearance of starch granules, unchanged or only slightly swollen, therefore indicates a much more severe grade of insufficiency of small intestinal digestion than does the appearance of muscle fibers.

After the ingestion of oily seeds such as almonds, in place of starch or with it, are seen granular structures of a rounded or angular form known as **aleurone grains.** These are albuminoid in nature and give no blue color with iodine. They are freely soluble in water and pass out of the cell in which they occur in the intestine. As they are insoluble in serum they are seen frequently in stools of serous diarrhea and dysentery.

Cellulose and vegetable tissue: Recognition of plant cells and cellulose is important in examination of stools because they may be mistaken for animal remnants and parasites. There is some obscurity as to the exact changes that take place in the process of digestion of vegetable matter. Plant cells are covered or enclosed with cellulose which is commonly supposed to be quite resistant to the action of digestive juices. This is not altogether true, for Moeller has shown that thin cellulose walls are readily broken down in the digestive tract; the pectinlike material that forms the middle lamellae of plant cells is attacked in the intestine and the cells fall apart and are thus exposed to further digestive activity. Cellulose found in stools is not chemically the same as when ingested, for, instead of the faint blue which they give with iodine and sulfuric acid before digestion, they show a faint purple coloration. They are also much more freely soluble in copper oxyammonia after digestion than they are before digestion. The nature of the change is probably a hydrolysis brought about by the action of bacteria, the alkaline contents of the intestine, and the intestinal enzymes. The enzymes from certain molds have the power of breaking down the toughest kind of cellulose, e.g., that of wood. The digestion of cellulose is aided by constipation because the bacteria have a longer period of contact under these circumstances, whereas in diarrheal states with rapid peristalsis the cellulose is passed almost unchanged. Modifications of cellulose, such as the cutinized peripheral walls of stems and leaves and corky cells, are much more difficult to digest than unaltered material. For this reason they are seen more often in stools. Flat epithelial cells with the intervening stomas of leaves and also plant hairs are characteristic. The plant detritus most often seen is the lignified cells forming the spiral pitted and scariform vessels and the vast fibers of the fibrovascular bundles. Young green vegetables give only spiral vessels, pitted and reticulated ducts appearing chiefly in old and overripe plants. Woody tissue, fruit envelopes of vegetables and fruits, pass through the intestinal canal practically unchanged and appear in stools as such.

CHEMICAL ANALYSIS

Fresh stools are usually used in chemical researches, but one may use dried stools satisfactorily. Chemical analysis is not commonly performed because of the time necessary for its completion and because not every case requires such an analysis. However, an occasional chemical analysis gives information to the internist which cannot well be otherwise obtained.

Reaction

Stools are ordinarily neutral in reaction, although acid and slightly alkaline stools apparently may occur under normal conditions. This acid reaction is due to the presence of lactic or butyric acid and usually is indicative of fermentation in the intestinal canal.

Litmus is the usual indicator. Moisten red and blue litmus paper with distilled water, place on a glass slide, and rub on a fragment of feces, observing the change of color from the other side. Or place a small amount of stool in a test tube, add sufficient water to make a suspension, and shake well. Into another small test tube introduce a few ml. of a watery solution of tincture of litmus and add a few drops of the fecal suspension. Observe the color change. If the cloudiness of the fecal suspension interferes with the reading, centrifuge the suspension first and use only the supernatant fluid.

A method published by Bruce[4] follows: Make a 1% solution of alizarin. Place 2 small drops of the indicator on a glass slide about 1½ in. apart. Dip a stirring rod into the fluid part of the specimen or, if the feces are formed, merely puncture the mass. Mix thoroughly in one of the drops, using the other drop as a control. An alkaline reaction is indicated by a reddish violet to violet color; neutral, no change; and acid, a light yellow. The density of the colors depends upon the amount of acid or alkali present. By placing the slide upon white porcelain, the depth of color may be more easily determined. After a few observations, it is easy to detect any abnormality in the reaction.

Changes in reaction: Meat diet gives an alkaline reaction; the more meat ingested, the stronger the alkalinity. Blood, mucus, and pus change the reaction. As putrefaction increases alkalinity and fermentative changes increase acidity, the reaction must be taken soon after passage of the stool. Excess fat increases acidity from the presence of fatty acids. Catarrh of the upper intestine increases the acidity if peristalsis is rapid. Chronic processes of this kind, however, produce an alkaline state, e.g., in chronic enteritis, whether tuberculous or amebic. If there is a block in the pancreatic juice outflow, there are fatty stools with marked acidity. The presence of much protein may be masked by the ammonia formed, giving an alkaline stool.

Reaction of infants' stools is dependent upon the diet. Breast-fed children have faintly acid stools. Bottle-fed children have neutral or slightly alkaline stools. The reaction is dependent upon the proportion of fat and protein present; i.e., when fat predominates, the reaction is acid; when protein predominates, it is alkaline. Therefore persistent alkaline stools from a breast-fed child may indicate that the mother's milk is deficient in fats.

"Dry" constituents of stools

Normally the stool consists of liquid parts, 65-85%, and dry solids, 15-35%. This is largely dependent upon diet, a meat diet increasing the solids and a vegetable diet increasing the liquid parts. Under pathologic conditions the relative proportions of dry to liquid constituents change; i.e., the dry part decreases when there is a block and the liquid constituents increase, the most extreme condition occurring in cholera, in which, according to the statistics of C. Schmidt, only 1-2% of dry substances is found. Increase in liquid constituents of stools is due to decreased resorption and increased secretions within the intestines. It might be termed a combination process, with the increase in transudation in the principal role. Schmidt believed that the extreme condition just noted, Asiatic cholera, is an example of blood transudation. An analogous condition obtains when senna or other purgatives are taken. The exact mechanics of these drugs is still in dispute, however.

Bile and biliary constituents

The pigment of feces which gives it its color is urobilin or stercobilin, formed from the bilirubin of the bile by reduction processes in the intestine. This conversion of bilirubin into urobilin does not take place in man until bacteria appear in the intestine in early infantile life.

Schmidt sublimate test for urobilin

Place a portion of fresh feces the size of a walnut in a mortar and rub up with a small quantity of concentrated solution of bichloride of mercury (25 gm. bichloride of mercury, 2.5 gm. sodium chloride, 500 ml. distilled water). Allow to stand for 24 hr. in a covered Petri dish. If urobilin is present, a deep red color develops in a few minutes. If only a trace is present, it may take 24 hr. to develop this color. This reaction may be obtained only if stools are quite fresh. Stools containing much fat must be extracted with ether before applying this test.

Schlesinger test

This is substantially the same as that for urobilirubin in urine.

Shake a sample of feces with ether to remove the fat. Extract the residue with acid alcohol (5 ml. hydrochloric acid and 95 ml. alcohol); neutralize the yellow fluid that results with ammonia or sodium hydroxide solution. Mix with an equal quantity of 10% zinc acetate in alcohol. Remove the precipitate, if any forms, by filtration through a dry filter paper. Examine the clear filtrate against a black background for the green fluorescence that indicates urobilin. Examine the red fluorescent solution with the spectroscope; it will show an absorption band between F and B which moves nearer to F when the fluid is acidified. No bands can be seen if feces contain blood.

These tests are used to determine whether stools are really acholic. A negative result with the zinc acetate test shows that no bile is reaching the intestine, and when associated with deep painless jaundice and progressive loss of weight and strength, is strongly suggestive of malignant disease causing obstruction of the biliary system.

Bilirubin alone or mixed with biliverdin is encountered normally only in stools in the first week or two of life.

Gmelin test for bilirubin

Add a few drops of nitric acid containing nitrous acid to a smear of feces on a piece of white porcelain; a play of colors, green, blue, violet, red, and yellow develops. If the stools are already green from the presence of chlorophyl or bacterial pigments, extract with water and test the aqueous extract.

Bilirubin is seen under pathologic conditions in diarrheal states, especially the jejunal diarrhea described by Nothnagel.

Bile acids, glycocholic and taurocholic, are completely reabsorbed from the intestine so that only a very small quantity appears in the lower part of the bowel. There, under the influence of putrefactive bacteria, they are split up into glycocoll, taurine, and cholalic acid. The cholalic acid combines to form cholate of soda which, under normal conditions, is the only representative of bile acids in the stool. If the intestinal contents pass so rapidly that the usual fission processes do not have time to take place within the large intestine, unaltered bile acids may be present.

Test for biliary acids

Extract a small quantity of feces with alcohol and filter. Distill the filtrate to drive off the alcohol, take up the residue with water rendered faintly alkaline with sodium hydroxide. Treat the watery solution with a trace of cane sugar or a few drops of a dilute solution of furfural, and add concentrated sulfuric acid. In the presence of bile acids a crimson color develops. The sulfuric acid must be added a drop at a time, shaking after each addition. Care must be taken that the temperature does not rise above 70° C., the test tube being cooled in water from time to time.

Watson test for urobilinogen

This is fully discussed on p. 1827.

Spectroscopic and chemical tests for occult blood

We have already discussed the presence of blood in the gross examination of stools, as well as its presence microscopically. Blood in extremely small amounts can be detected as occult blood by chemical or spectroscopic methods. These tests all depend upon the detection of one of the constituents of blood, hemoglobin.

The finding of occult blood in feces is of the greatest importance in the diagnosis of ulceration of the digestive canal. Hemorrhage from the nose, throat, esophagus, and hemorrhoids must be excluded. The finding of occult blood may establish whether an ulcer in the digestive tract is or is not healing.

Significance of occult blood: One must be guarded in the interpretation of a positive benzidine test for blood because it is difficult to differentiate exogenous from endogenous blood. The smallest amount of meat in the diet is likely to give a positive benzidine blood reaction. The patient must be kept on a meat-free diet for at least 3 days before making this test if the results are to be meaningful. The physician who interprets this test must make certain that there has been no bleeding from the hemorrhoidal vein. A positive reaction means bleeding from any portion of the tract. Blood may be found in either benign or malignant disease. During the suckling period blood is encountered in melena vera, hemorrhagic enteritis, from fissures, polyps, rectal papilloma, trauma, the hemorrhagic diatheses, invagination, and typhoid perforation. Duodenal ulcers occasionally occur in children and of course may lead to bleeding.

Spectroscopic test

Snapper's method is the best.

1. Extract the feces in a mortar with acetone to remove the coloring matter. This leaves a dry mass.
2. Transfer the dry residue to a clean mortar and rub up with a mixture of 1 part glacial acetic acid and 3 parts ether, preferably ethyl acetate.
3. Filter. Add ¼ vol. pyridine, and then a drop of ammonium sulfide solution.
4. Examine spectroscopically. If blood is present, a hemochromogen band will be found on the boundary between yellow and green.

Chemical tests

The chemical tests are simpler. These are the guaiac, aloin, benzidine, and others.

Boas-Weber test

1. Mix 1 gm. feces with 10 ml. 70% chloral hydrate in 95% alcohol.
2. Add 1 ml. glacial acetic acid and filter.
3. To 1 ml. filtrate add 1 ml. 2% guaiac resin in equal parts of chloral hydrate solution and hydrogen peroxide (3%).

A **blue** color indicates blood.

Kuttner-Guttmann modification of Weber test

1. Stir 8 gm. feces with acetone. Let stand and pour off the supernatant fluid.
2. Treat the residue with 5 ml. glacial acetic acid in 10 ml. 7% sodium chloride solution.
3. Extract with ether by adding the ether to the mixture, allowing it to settle, and then decanting the ether.
4. Add a few drops of glacial acetic acid to the ether.
   (a) Mix 1 ml. of this with 2% guaiac resin,
   (b) Mix 1 ml. of this with 0.2% guaiac resin,
   (c) Mix 1 ml. of this with 0.02% guaiac resin.

All 3 resins are dissolved in equal parts of alcohol and oil of turpentine.

The solution becomes **purple** if blood is present.

The different concentrations are necessary because one cannot tell how much blood is present before making the tests.

Objection to the test: Less than 10% of blood in feces will not show a reaction.

Boas phenolphthalein test

Reagent:

Phenolphthalein	1 gm.
Potassium hydroxide	25 gm.
Distilled water	100 ml.
Zinc powder	10 gm.
Hydrogen peroxide, 3%	5 drops
Alcohol	2 ml.

Procedure:

1. Stir 1 gm. feces in a mortar with 5-6 drops glacial acetic acid and 10 ml. alcohol.
2. Filter.
3. Pour the filtrate on a mixture of 15 drops Boas phenolphthalein reagent.

A **red** color denotes blood.

Boas thymolphthalein test

Reagent:

Thymolphthalein	10 gm.
Potassium hydroxide	1 gm.
Distilled water	25 gm.
Zinc powder	100 ml.

Procedure:

1. Stir 1 gm. feces with 10 ml. alcohol and 10 drops glacial acetic acid.
2. Filter.
3. To 30 drops filtrate add 30 drops Boas thymolphthalein reagent and 15 drops 3% hydrogen peroxide.

A **blue** color denotes blood.

Benzidine test—Levin-Watt

To make the benzidine reagent dissolve 6 gm. benzidine base, c.p., powder in 100 ml. 95% ethyl alcohol and preferably allow to age 2 or 3 days before using.

1. Collect fecal material in clean containers. Emulsify a small portion of the feces in distilled water; then pass through filter paper (Whatman No. 5 or more retentive paper) into a clean test tube to obtain a clear filtrate. If clouded due to breach in filter paper, refilter.
2. To 3 ml. filtrate add 8 drops 50% aqueous acetic acid or glacial acetic acid, with mixing.
3. Add 8 drops hydrogen peroxide (c.p., 3%) to the resulting mixture and shake.
4. Overlay the final mixture with an alcoholic benzidine solution, drop by drop, to obtain a contact ring. Tilt to bring about slight mixing at the ring. A positive reaction is indicated by the appearance of a greenish ring at the area of contact of the benzidine solution and the filtrate mixture, spreading into the benzidine solution either immediately or up to 2 min. time, the intensity or gradient of color being in relation to the concentration of blood.

When testing for blood in vomitus and gastric contents, add water in half the volume of material used, filter, and carry through the test as previously. In urine, when only a few red blood cells are found microscopically, centrifuge or sediment the specimen. Transfer the bottom 0.1 ml. containing the sediment, to a clean test tube, add 0.4 ml. distilled water, and continue as previously described.

This method eliminates most of the substances producing false positive reactions as follows: (1) filtration of the stool suspension eliminates blood-containing particles of meats, oxidase-containing particles of foods, particles of animal charcoal, and insoluble metal salts (of iron, copper, etc.) and (2) the acetic acid destroys soluble oxidases. In the contact layer or ring test the soluble hemoglobin compounds give true positive blood reactions in dilutions up to 1:100,000; meat-free days are unnecessary preceding this test for occult blood.

Benzidine dihydrochloride test

This is described on p. 1835.

Hematest

See p. 1841.

Protein substances

Coagulated albumin is not found in stools under normal conditions. Soluble albumin and proteoses are present only under pathologic conditions. Inasmuch as nucleoprotein is always present, being derived from the nuclei of the mucosa and food remnants, always remove it before testing for albumin.

Biuret test for albumin

1. Mix the feces with water to a consistency of thin syrup. Test with litmus paper and add to each 10 ml. a quantity of 10% acetic acid and alcohol, varying with the reaction: if the stool is strongly acid, add 0.15 ml.; if faintly acid or neutral, 1 ml.; if slightly alkaline, 1.5 ml.; if strongly alkaline, 2-2.5 ml. Mix well.
2. Rub 5 ml. chloroform into the mixture. Place in a test tube and let stand until the chloroform and the larger particles sink to the bottom.
3. Decant the upper yellow and faintly tinted fluid into another test tube and add a disk of copper sulfate agar. After 1 hr. take out the disk, wash with water, and examine.

If the feces contain a large amount of albumin, the disk retains its bright blue color, but if no albumin or only a trace is present, it will be brownish blue. On pouring over it a little diluted solution of potassium hydroxide, the color will change to blue violet if albumin is present.

Copper sulfate agar is made by boiling 2 gm. agar in 100 ml. distilled water until dissolved, and then adding 10 ml. 10% solution of copper sulfate. Pour the hot mixture into a glass tube 0.8-1 cm. in diameter, 20-30 cm. long, closed at one end by a plunger. Close the other end with a rubber cap. To make a test remove cap, push in the plunger, and cut off a disk about 1 cm. long.

Caution: Urobilin also gives the reaction. Ury noted that other pigments and the remnants of paranuclein and casein give misleading results.

A simple test will be found on p. 1818.

Dissolved albumin is found pathologically only with inflammatory conditions. Proteoses are found only under rare conditions and always in association with albumin. They are found more frequently in stools of babies, but here they undoubtedly arise from the food ingested and not from the intestinal wall.

Casein: No satisfactory test for casein has been devised. It is better to rely upon macroscopic test than upon chemical analyses.

Mucin: Mucin is a compound protein radical and a nitrogen-containing carbohydrate, glucosamin. Boiling with acids makes mucin yield a copper-reducing substance like a sugar. Mucin dissolves in weak alkaline solutions and is precipitated by acetic acid. It may be identified by acetic acid, with microscopic study. The mucin becomes cloudy and develops lines. This is a better method than the chemical methods.

Protein derivatives

Amino acids: The digestion and putrefaction of protein substances in the intestine yields a number of amino acids. Among these are leucine (amino-isobutyl acetic acid), tyrosine (oxyphenyl-amino-propionic acid), and tryptophan (indole-amino-propionic acid). These undergo further changes, breaking down into phenol, paracresol, indole, and skatole, which appear partly in urine with sulfuric and glycuronic acids and partly in feces in an unchanged state. Lack of pancreatic action causes leucine and tyrosine to appear in feces.

Gelatin film test for stool trypsin—Schwachman et al.

The gelatin film test estimates roughly the proteolytic activity of stools by determining their ability to liquefy gelatin. Fecal suspensions are placed upon gelatin film (unexposed and unfixed), and the extent of removal of the gelatin film is noted.

In determining the significance of the test, Schwachman et al.[6] studied 2 groups of patients: (1) 500 normal and sick individuals and (2) 50 patients with pancreatic fibrosis. In group 1, less than 5% of the infant group lacked this proteolytic power. As one advanced into the older age groups, this percentage increased. The administration of a laxative for some older children without tryptic activity resulted in stools possessing tryptic activity.

A total of 220 separate stool specimens was examined from patients in group 2. In 209 specimens there was no tryptic activity. The remaining 11 specimens from 7 patients showed definite tryptic activity. In this group 3 patients had minimal tryptic activity in the duodenal fluid.

When oral pancreatin was given to patients with pancreatic fibrosis, the stools became positive for trypsin. When laxatives were given to a few patients with pancreatic fibrosis, the stool trypsin remained negative.

Conclusions and interpretations based on a single examination are hazardous. At least 3 specimens should be studied to eliminate or to make probable the diagnosis of pancreatic insufficiency, and whenever doubt arises, duodenal intubation is indicated.

There are 2 procedures, a test tube method and a gelatin film method. The test tube method is more reliable and reproducible because the film substrate in the gelatin film method might change its properties when exposed to different temperatures and if stored for varying periods of time. However, the film method offers the advantage of simplicity and convenience and can be used as an office test. Photofilm is less reliable than gelatin film.

(1) Test tube method

Reagents:

1. Sodium bicarbonate solution, 5%.
   - (a) Dissolve 5 gm. sodium bicarbonate ($NaHCO_3$) in distilled water and dilute to 100 ml. with distilled water.
2. Gelatin solution, 7.5%.
   - (a) Dissolve 7.5 gm. gelatin in 100 ml. distilled water heated to about 37° C.

Procedure:

1. Place 4 ml. distilled water in a test tube graduated at 5 ml.
2. Add feces to the 5 ml. mark.
3. In another test tube graduated at 10 ml., place 9 ml. distilled water.
4. Add feces to the 10 ml. mark.

This gives two samples of fecal material diluted, respectively, 1:5 and 1:10 with distilled water. Further dilutions can be made as desired.

5. Mix the contents of the 2 tubes thoroughly.
6. Place 1 ml. of 1:5 dilution in one tube and 1 ml. of 1:10 dilution of feces in another tube.
7. Add 1 ml. 5% sodium bicarbonate and 2 ml. 7.5% gelatin solution, previously warmed to 37° C., to each of the 2 tubes.
8. Mix well and place the tubes in a 37° C. water bath for 1 hr.
9. Then transfer both to an ice bath for 30 min.
10. At the same time prepare a control tube containing all the ingredients except the feces and carry it through the same procedure.
11. Examine all tubes at the end of the 30 min. period of ice bath incubation.

If the contents remain liquid, record the tryptic activity as 4+. A rigid gel indicates no activity. Compare the tests with the control.

(2) Gelatin film method

1. Prepare stool dilutions in water as described in method 1.
2. Place 1 large drop (approximately 0.1 ml.) of the well-mixed specimen on an unexposed,

unfixed gelatin film such as is used in routine hospital practice. The film strip may be clipped to a cardboard to prevent curling.

3. Incubate the film at 37° C. for 1 hr. Although 37° C. is the recommended temperature, room temperature is satisfactory if the incubation period is prolonged to 1½-2 hr.
4. Wash the film in a stream of cold water with gentle rubbing.

The result is now apparent. A clearing at the site of the drop indicates enzyme activity. A complete clearing is designated as 4+, and a slight clearing only at the periphery of the drop as 1+.

If the drop is small or if the concentration of the specimen is too great, the material will dry and cake.

Proper bacteriologic precautions should be taken when dealing with cases suspected of enteric infections.

Both a 1:5 and a 1:10 dilution of stool are used routinely.

When performing the test for the first time or when using new gelatin film, adequate controls should be run. Determination of the end point using normal infant stools or a standard trypsin solution is suggested.

Discrepancies between the film and tube methods occur in less than 1% of the tests. The tube method is more sensitive. For example, a specimen showing a 2+ reaction in the 1:10 dilution and a 4+ reaction in the 1:5 dilution on the film will be strongly positive by the test tube procedure using a 1:10 dilution.

Fischer-Bergell test for amino acids

Rub up feces with water and then with acetic acid; boil and filter. Concentrate on the water bath, cool, and shake 8 hr. with ether in a shaking machine to remove the fat. Decant the ethereal portion and mix the residue with potassium hydroxide; add 25% sulfuric acid and 20% phosphotungstic acid. Filter. Add barium hydroxide to the filtrate, concentrate it on the water bath, and for every 500 ml. of the original fluid add 2 ml. 10% solution of naphthalene-sulfo-chloride. Make slightly alkaline and shake for 12 hr. in the shaking machine. Every 3 hr. add 1 ml. reagent and a little potassium hydroxide. Flood the product at the end of the shaking with ether, acidify the supernatant fluid, and allow to stand 24 hr. At the end of that time a crystalline deposit of naphthalene-sulfo-tyrosine is formed. It is readily crystallizable from alcohol, melting at 129° C. The watery portion gives a strong Millon reaction.

Fat

In a consideration of fat in stools we refer actually to the higher insoluble fatty acids. To the casual reader the fat in stools is closely related to the fat ingested and the ability or lack of ability of the normal physiologic processes to handle it. This is true in the main; yet, according to Williams' researches, there may be a general metabolic disorder that throws on the intestine the onus of eliminating some of the fatty acids, calcium salts, etc.; i.e., some of this fat is endogenous and not exogenous The most important conclusion to be drawn from the presence of a large amount of fat in stools is a block in the outflow of bile into the intestinal canal.

According to Biebert, the presence of more than 40% of fat in stools indicates a condition of fat diarrhea. In normal dried stools of infants the fat should not exceed 20%. Increase in fat content to a marked extent is always a biliary or pancreatic affair. Pancreatic juice has for a principal function the digestion of fats, which is markedly increased by the presence of bile and still more by the presence of both bile and hydrochloric acid. Bile of itself can do nothing with fat, but a mixture of bile and pancreatic juice can digest 3 times as much fat as pancreatic juice alone. Wohlgemuth states that the lipase of the pancreatic fluid exists in an inactive form and that it is activated by the bile. The bile also helps to dissolve the free fatty acids formed by the action of the pancreatic lipase on neutral fats, for these acids are insoluble in water but very soluble in bile: bile therefore assists in the absorption of fats.

No data of any value can be obtained from a chemical analysis for fat unless dried feces are used.

Place a required quantity of feces in a shallow porcelain dish of known weight and carefully weigh. Transfer the dish and contents to a water bath kept at 50°-60° C. After most of the water has been driven off, break up the residue with a glass rod. Return the dish to the water bath and heat for another 30 min. Remove the feces from the dish, transfer to a porcelain or glass mortar, and grind to a fine powder. Return the powder to the dish and heat for another 30 min. Cool the dish and weigh, repeating the process. Two consecutive weighings must give the same results. This figure gives the weight of the dried feces from which to deduce the water content. If the stool contains much fat, it is difficult to dry it because the fat forms a film on the surface which interferes with evaporation. To avoid this, lower the boiling point by the addition of alcohol from time to time. Heat on the water bath for 3-4 hr. in a porcelain casserole. Add 50 ml. absolute alcohol, stir with a glass rod, and again heat. In 1 hr. add 25 ml. more alcohol, stirring well, and evaporate as before. Add a third portion of alcohol, stir, and evaporate for another hour. Heat on the water bath at 108° C. for a short time, thus removing all the water. When a constant weight is obtained, there is no more water present.

Another rapid method of drying for fat estimation only: Mix the stool with 10 vol. of silver sand

(Merck's purified sea sand) which has been successively washed in hydrochloric acid, water, and alcohol and dried. Dry at 100° C.

Test for total fat

Neutral fats and free fatty acids are soluble in ether; combined fatty acids or soaps are not. To include both in the analysis, one must break down the latter and set free the fatty acids with a mineral acid.

1. Place 0.5 gm. dry powdered feces in the lower bulb of the Schmidt-Werner milk tube with the use of a funnel.
2. Pour 10 ml. diluted 30% hydrochloric acid into the tube to carry down the particles that may adhere to the sides.
3. Heat in a boiling water bath for 15 min., rotating occasionally to mix contents. Cool, fill to the 50 ml. mark with ether, securely cork, invert 40 or 50 times, rotate between hands to get rid of air bubbles, fix in upright position, and allow to stand a half hour to give time for all the solid particles to settle to the bottom of the tube. Rotation of the tube occasionally hastens this process.
4. Pipet off 20 ml. of the clear supernatant fluid and place in small weighed beaker. Evaporate the ether, dry the residue on a water bath, cool, and weigh.
5. The difference in weights is the weight of the fat contained in 20 ml. of the ether. Read off the height of the liquid in the tube after the 20 ml. have been withdrawn.

Calculate the percentage of fat as follows:

If w = the weight of the feces taken
x = the weight of fat found in 20 ml. ether
y = the number of ml. left in the tube

then (y − 10) will be the amount of ether left in the tube, for 10 ml. were dilute hydrochloric acid, and 20 + (y − 10) = (y + 10) will be the total bulk of the ether in which the fat from "w" grams of feces was dissolved.

$$\frac{x\ (y + 10) \times 100}{20 \times w} = \text{percentage of fat in stool}$$

Besides the total fat, the ether extract of the feces contains the lower fatty acids, cholesterol, lecithin, cholalic acid, pigments, etc. These exist in quantities so small that they are negligible.

Tests for neutral fat, free fatty acids, and soaps

Neutral fat and free fatty acids are soluble in ether, whereas the combined fatty acids or soaps are not and must be broken down by the addition of a mineral acid to make them soluble.

Test for **neutral fats** in the following manner:

1. Place 0.5 gm. dried feces in the lower bulb of the Schmidt-Werner tube; add 10 ml. distilled water.
2. Heat the tube for 15 min. in a boiling water bath, rotating the tube occasionally to get a good mixture.
3. Cool; fill to the 50 ml. mark with ether; carry on the remainder of the test of the feces for total fat and the fatty acids and neutral fats side by side, labeling the tube "A" that has been treated with acid,* and the tube "B" that has been treated with water. The ether extract of tube B gives the sum of the neutral fats and the fatty acids (free). The contents of tube B gives the unsaponified or, as Cammidge speaks of it, the "unsoaped fats," whereas the difference between the 2 gives the combined fatty acids or soaps.
4. To determine the relations between the neutral fats and free fatty acids, dissolve the ether extract from tube B in an excess of ether (about 20 ml.), add a few drops of an alcoholic solution of phenolphthalein; then titrate with N/10 sodium hydroxide in alcohol.

The number of ml. used to neutralize the solution multiplied by 0.0284 gives the weight of the free fatty acids contained in the quantity of feces originally used. The difference between this and the weight of the ether extract gives the quantity of neutral fat (and lipoids).

There are a number of other tests for fat that are sufficiently important to be carried out in certain cases.

The **acid test** is the number of mg. potassium hydroxide necessary to neutralize 1 gm. fat; this is a measure of the degree to which the fat has been hydrolyzed. Weigh a small amount of the fat, dissolve in neutral alcohol or alcohol-ether; add a few drops 1% alcoholic phenolphthalein, or if the fat is dark colored, a 2% solution of alkali blue 6 B; titrate with N/10 or N/2 aqueous potassium hydroxide.

The **saponification value** is obtained by determining the number of mg. potassium hydroxide necessary to neutralize the fatty acids split off from 1 gm. fat on saponification.

1. Boil a weighed quantity of fat with alcoholic potassium hydroxide on a water bath for 30 min.
2. Titrate the alkali in the flask with 0.5N hydrochloric acid, using phenolphthalein as an indicator. Compare the result with that given by a control experiment in which the alcoholic potassium hydroxide only was used. The difference gives the amount of alkali neutralized by the fatty acids liberated from the weighed quantity of fat, and from this the saponification value can be calculated. As the soaps formed are hydrolyzed by water and therefore react alkaline, it is necessary to add neutral alcohol from time to time in sufficient amount to keep it at over 40% since hydrolysis of soaps does not occur in this strength of alcohol.

The saponification value is a measure of the mean molecular weight of the fatty acids entering into the composition of the fats. By means of it the nature of the fat present is suggested. Fats such as tallow or lard give a saponification value of about 195, butter 220-233, cocoanut oil 250, and cod liver oil about 171-189.

The **ester value** is the difference between the saponification and the acid values of the fat and is therefore the number of mg. potassium hydroxide necessary to neutralize the fatty acids present in the form of neutral esters.

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*Test for total fat.

The **iodine value** of a fat is the amount of halogen, expressed as iodine, that the unsaturated fatty acids of this fat will absorb, expressed as a percentage of the weight of the fat. The iodine value of a fat or a mixture of fats is therefore an index of the proportion of unsaturated or saturated fats present, for the latter do not absorb iodine. In feces, oleic acid, with an iodine value of 90.1, is the most important unsaturated fatty acid. Feces of children fed on cow's milk have a lower iodine value and a higher melting point than of those fed on mother's milk. The first stools of the latter after the meconium has been passed are particularly rich in this substance. They have an iodine value of 48% and a melting point of 40° C. At the end on the first week the proportion of oleic acid falls, and the melting point and iodine value approximate those of older children; namely, 47° C. for the former and 20% for the latter. In cases of dyspepsia the iodine value rises and the melting point falls.

Method: A titrated solution of iodine monochloride, obtained by mixing solutions of iodine trichloride and iodine in glacial acetic acid, is added to a solution of the fat in carbon tetrachloride. This, after standing in the dark for 1-2 hr., is mixed with a 10% solution of potassium iodide and is then titrated with a standard solution of sodium thiosulfate. From the amount of iodine absorbed by the known weight of fat taken the iodine value can then be calculated.

Hehner's value is the percentage of fatty acids insoluble in water upon saponification of the fat. For triolein, the Hehner value is 95.7; for lard and most fats and oils, 95; for butter, between 86 and 88; and for cocoanut oil, still lower.

Saponify a weighed quantity of fat with alcoholic potassium hydroxide, evaporate the alcohol, dissolve the soap in hot water, and make the solution acid with hydrochloric acid. Heat until the aqueous layer is clear; then filter. Wash the fatty acid with boiling water as long as any acid reaction can be detected; then dry and weigh.

The **Reichert-Meissel value** is the number of ml. of N/10 potassium hydroxide necessary to neutralize the volatile acids produced by the saponification of 5 gm. fat and is a measure of the amount of lower molecular weight fatty acids (which volatilize in a current of steam) that enter into the composition of the fat. To obtain comparative values the experiment must be carried out under certain prescribed conditions, which vary somewhat in different countries.

The **acetyl values** gives the number of mg. potassium hydroxide necessary to neutralize the acetic acid liberated when 1 gm. acetylated fat or fatty acids, formed by heating the fat with twice its weight of acetic anhydride, is saponified. Animal fats do not give an acetyl value unless they are mixed with cholesterol or some alcohol soluble in water, but certain vegetable oils have high acetyl values; thus for castor oil it lies between 153 and 156.

Total nitrogen

Use the Kjeldahl method. The ammonia nitrogen and protein nitrogen are converted into ammonium sulfate by heating with sulfuric acid in the presence of a catalyst. The ammonia is set free with an alkali, and the steam distillate is titrated with a standard acid. Use dry stool or about twice the weight of fresh stool.

Weigh 2 gm. dried feces and place in the bottom of a Kjeldahl flask.

Add 800 ml. pure sulfuric acid, 200 ml. fuming sulfuric acid, and 100 gm. of anhydrous phosphoric acid free from nitrogen. Add about 0.1 ml. mercury from a pipet; place flask aside for 12 hr. after shaking it, as it will foam too much if heated at once. Heat in the usual inclined position on a sand bath or asbestos mat with a small flame, and later with a full flame, until the fluid is clear. This takes 3-4 hr. Shake down any particle that comes up on the neck of the flask. Cool and wash the contents into a 500 ml. distillation flask with 50 ml. water. Wash the residue into this flask with fresh portions of water, keeping the total contents below 200 ml. Cool the flask, add 50 ml. 10% sodium hydroxide solution, 40 ml. 4% potassium sulfate solution, and a spoonful of talc or a few zinc filings. Add 60 ml. sodium hydroxide solution; connect quickly with the condenser. The delivery end of the condenser dips into a small flask containing 50 ml. N/5 sulfuric acid. Heat and distill. Disconnect and stop the heating in 20 min. Wash the delivery tube with a little distilled water into the receiving flask, adding a little cochineal or methyl orange solution. Titrate with N/5 sodium hydroxide. The number of mg. nitrogen in the dried stool is obtained by multiplying the number of ml. of N/5 sulfuric acid bound by the distilled ammonia by 2.8.

An alternate method is described on p. 41.

The interpretation of the amount of total nitrogen found is difficult: The nitrogen comes from the proteins of food, from the secretions and metabolism of the gastrointestinal tract, and from bacteria. Schmidt and Strassburger consider that on a diet yielding only a small amount of residue, bacteria furnish no less than ¼ or even ½ of the total nitrogen of stools. In general, very little help is obtained in the diagnosis of pathologic conditions from total nitrogen determinations.

Carbohydrates

Sugars do not ordinarily appear in stools of adults. Interference with absorption may cause them to appear in stools of children. Sugars can be determined by the usual reduction processes. It is more important to determine, however, whether the normal process of fission has taken place. Orban proved the absence of lactose in the acute stages of several intestinal affections of children, and Pfaundler found that the inversion of milk sugar does not take place in marasmic children, thus accounting for the lactosuria that has been reported in such cases.

Extract the dried stool with water; filter; test the filtrate, with or without evaporation, with Fehling, Trommer, Nylander, phenylhydrazin, or

Benedict reagent. Proteoses and peptones are interfering substances; therefore, it is preferable to extract with alcohol, filter, and evaporate the alcohol, dissolve residue in water, and apply the reduction tests.

Starch may best be determined by the microscopic or the microchemical methods already described.

Of pure chemical methods we shall describe the well-known Schmidt fermentation test. This test depends upon the fact that the dissolved carbohydrates, as well as those starches that lie free and are easily acted upon, are inverted by the diastase which is always present in the feces and are then fermented by the intestinal bacteria, with the formation of gas.

Procedure: Place 5 gm. stool, well mixed with water, in the receptacle of the Schmidt apparatus and close with a rubber stopper, excluding air bubbles. Connect the outlet tube with a closed graduated tube filled with water in which the gas that is formed collects. Join this in turn to a tube where the displaced water passes. Fill and set up and place in the incubator at 37° C. for 24 hr. If gas has collected in the tube, cool the apparatus to the temperature of the room and measure the gas. Test the reaction of the fecal mixture with litmus. An acid reaction plus gas indicates the presence of fermentable carbohydrates. Only a positive result is of value.

Schmidt claims that a patient should take a test diet for several days, and if the gas fills ¼ of the tube, intestinal dyspepsia can be proved. Rice, cellulose, and fat should be excluded from the diet.

Concretions

Our interest in the various kinds of concretions found in stools lies mainly in distinguishing coproliths from real concretions such as gallstones, pancreatic calculi, and intestinal sand. It is comparatively simple to distinguish between a stone composed of organic material from one of inorganic structure; heat on a platinum dish causes the organic material to carbonize and crumble away, while the inorganic substance remains unchanged or blackens a trifle. Furthermore, organic pancreatic stones, according to Minnich, on heating give off an aromatic odor and are easily soluble in chloroform.

Gallstones: Gallstones consist of a mixture of the constituents of the fluid in which they are produced. They are always "mixed"; that is, they have more than one constituent. Cholesterol is found in all stones. Calcium salts of the bile pigments are commonly present; sometimes they form the chief part of the stone. Naunyn divided them into the following classes: pure cholesterol stones, laminated cholesterol stones, common gallbladder stones, mixed bilirubin-calcium calculi, and rare forms.

Pure cholesterol stones: These are smooth, white to yellow, rarely green or brown on the surface, crystalline, oval or spherical, and seldom faceted.

Laminated cholesterol stones: These contain calcium salts of the bile pigments, found in yellow, brown, or green layers, alternating with the white layers of cholesterol. Cholesterin forms 75% of the stone. They are often faceted.

Common gallbladder stones: These are chemically like the former; physically they show an external firm laminated crystalline crust which surrounds a softer pigmented nucleus, frequently containing a cavity filled with a yellow alkaline fluid. They are seldom larger than a cherry. Surface is yellow, sometimes white or brown. They are usually faceted.

Mixed bilirubin-calcium calculi: Calcium bilirubinate is the chief constituent, but there is also found biliverdin, calcium bilifuscin, oxidized bilirubin, and always some cholesterol. These stones are small and occur in 2 forms, one of waxlike consistency, with round irregular surface, the other hard and brittle, steel gray in color, with a metallic luster.

Rare forms: Rare forms are found, to wit, amorphous and incompletely crystalline cholesterol gravel with a nucleus of calcium bilirubinate, surrounded by cholesterol, varying in size from a grain of sand to a pea; calcareous stones consisting of calcium carbonate with calcium bilirubinate, rare in man, but common in cattle and herbivorous animals; concretions with included foreign bodies such as ligatures, needles, plum stones, around which gallstones are formed.

Test for stones

We have already noted the test in a platinum dish. Boil 2 gm. powdered stone in a flask with distilled water to remove the bile acids and other water-soluble substances. Divide the filtered extract into 2 parts. (1) Evaporate one to dryness and weigh, giving the weight of the water-soluble material in the amount of dry material used. Incinerate and again weigh, this weight being the quantity of water-soluble ash. Evaporate the other half (2) to dryness and extract the sodium glycocholate with a mixture of alcohol and ether from the dry residue.

Dry the residue from the water extract and extract with warm alcohol, thus separating the fats along with a small proportion of the cholesterol. Extract the residue with ether, which dissolves the cholesterol. Evaporate the ether, weigh the cholesterol, and examine for crystals of cholesterol, which are best seen by recrystallizing from warm alcohol; also try the chemical reactions for cholesterol.

1. Crystals treated with sulfuric acid (5 parts of concentrated sulfuric acid with 1 part water) melt at the edges and turn crimson. Add a small quantity of iodine and note a play of colors: violet, blue, green, and red. Evaporate a small quantity on the edge of a porcelain crucible with hydrochloric acid and add a trace of ferric chloride; this gives a blue color.
2. Dry cholesterol dissolved in chloroform and mixed with an equal volume of concentrated sulfuric acid gives, on shaking, a blood red upper layer of chloroform, which changes to cherry red and finally to purple, and a lower green fluorescent layer of sulfuric acid. With dilute solutions the chloroform solution is yellow to rose red, whereas the sulfuric acid is yellow with a greenish fluorescence.
3. A small quantity of cholesterol mixed with acetic anhydride gives a rose color changing to blue and then to blue green on addding concentrated sulfuric acid drop by drop (**Liebermann-Buchard** reaction). A specimen, purified by repeated crystallization from hot absolute alcohol and dried, melts at 145° C.

After treatment with ether, extract the residue with dilute hydrochloric acid (1:3) and filter. (Calcium, if present, is shown by making a portion of the solution alkaline with ammonia and adding acetic acid and ammonium oxalate solution. Copper may be detected by adding a few drops of potassium ferrocyanide, when a brown color develops.)

1. Wash the residue on the filter with water until free from hydrochloric acid. Dry the filter, cut into pieces, and warm in a dry flask with chloroform. Filter and examine for bilirubin, evaporate a small portion spontaneously on a watch glass, showing microscopically small, ill-defined rhombic plates, or indistinct crystalline grains which on being moistened with nitric acid give the colors of Gmelin reaction (p. 1957). Again shake the chloroform mixture with dilute sodium hydroxide; the pigment goes into alkaline solution, whereas the chloroform remains decolorized. The separated alkaline solution left to stand in the air turns green from the formation of biliverdin. Detect the presence of urobilin by the spectroscope.

Examine the stools carefully for stones, especially after washing in a sieve. Many of the objects commonly called gallstones are not real stones; they are intestinal sand coproliths. Chemical and microscopic study of all stones must be made to verify the diagnosis. With the aid of the roentgen ray, too, much information about the gallbladder region is obtainable which will substantiate the stool findings. An artifact is sometimes seen after the administration of large quantities of olive oil in suspected cholelithiasis. It is a concretion composed of fatty acids, fats, and soaps; it is soft, greasy, and transparent. Microscopic and chemical examinations will reveal its true nature.

Pancreatic calculi are rarely found, less than a hundred cases having been reported. They are small, gray white, with no facets on the surface. Heated on a platinum dish they yield a large amount of white ash and give off an aromatic odor, as previously stated. They are soluble in chloroform. Chemically they consist of 50% lime, as carbonates, phosphates, or even oxalates. Pigments and cholesterol in a very small amount are found in the external layers. Cammidge presents a very good illustration of these stones.

Intestinal sand has been previously described.

BACTERIOLOGIC EXAMINATION

When bacteriologic methods are applied to a study of stools, a never-ending task is encountered. The variety and number of bacteria normally found in stools are so great that ordinarily, unless there is some special reason, we do not make a thorough bacteriologic test. There are certain occasions, however, when a bacteriologic study must be made. Some mathematically inclined bacteriologist has shown that a normal individual passes out via the stool every day some 126 billion bacteria of many varieties and kinds.

Detailed methods for bacteriologic examination of the feces are found in Chapter 31.

In searching for bacteria, feces should be examined as soon after passage as possible. They should be passed in a sterile vessel, and rapidly smeared, stained, plated, and inoculated on special media or placed at once in a preserving or transport medium.

References for feces

1. Hazeltine, H. E.: U.S.P.H.S. Reports **32:** No. 48, 1917.
2. Greene: Medical diagnosis, Philadelphia, 1926, P. Blakiston's Son & Co., p. 865.
3. Hecht: Wien. klin. Wchnschr., No. 45, 1908.
4. Bruce, W. J.: J. Lab. & Clin. Med. **5:**61, 1919.
5. Levin, M. B., and Watt, J. Y. C.: Rev. Gastroenterol. **16:**650, 1949.
6. Schwachman, H., Patterson, P. R., and Laguna, J.: Pediatrics **4:**222, 1949.
7. Cammidge, P. J.: The faeces of children and adults, New York, 1914, William Wood & Co.

PART VII

BASAL METABOLISM AND ELECTROCARDIOGRAPHY

SECTION ONE BASAL METABOLISM

Chapter 109 Apparatus for measuring basal metabolic rate

SECTION TWO ELECTROCARDIOGRAPHY

Chapter 110 Use of the electrocardiograph
111 Unipolar lead electrocardiography
112 Interpretation of the electrocardiogram

Section One

BASAL METABOLISM

The terms basal metbolism and basal metabolic rate (BMR) refer to the heat production of an organism at the lowest level of cell chemistry in an inactive, awake, fasting state. This may be determined directly by means of a calorimeter or indirectly by calculating the heat production from an analysis of the end products of oxidation within the organism or from the amount of oxygen utilized.

Basal metabolism determinations are primarily used to evaluate thyroid function. Other laboratory tests that reflect thyroid activity are the measurement of serum protein-bound iodine, radioactive iodine uptake, and serum cholesterol. Elevated basal metabolic rates are seen in patients with toxic goiter. If any degree of thyrotoxicosis is present, tests done after 5-10 days of iodide therapy will reveal a lowering of the BMR by 15% or more.

Conditions other than hyperthyroidism that are frequently associated with an elevated BMR are pregnancy, hyperpituitarism, pheochromocytoma, polycythemia, and leukemia.

Diseases other than hypothyroidism that are often associated with a low BMR are Addison's disease, myotonia atrophica, and the nephrotic syndrome.

Fever increases the basal metabolic rate about 5% per degree. If the patient is febrile, it is preferable to postpone the test until the temperature has returned to normal.

Normal range: –10% to +15%.

GENERAL INSTRUCTIONS

The patient is instructed to eat or drink nothing except water after midnight prior to the morning of the test. If the patient is in the hospital, as soon as possible after awakening he is wheeled into the room in which the test is to be performed. Undue exertion is avoided.

If the patient reports to the laboratory for the test, he must rest for 30-60 min. During this time he is not to be disturbed by conversation, loud noises, or strong lights.

The basal metabolism apparatus should be prepared before the patient enters the room. Do not set up the machine in the patient's presence.

The procedure should be briefly explained to the patient before the rest period begins. It is necessary to explain that he is to breathe naturally into the apparatus and that the technician is simply going to measure the quantity of oxygen that is consumed. Frequently it is advisable to make 1 or 2 preliminary tests to acquaint the patient with the procedure.

The mouthpieces should be washed with warm water and soap, thoroughly rinsed, and then placed in boiling water for 30 min. They are then dried and stored. Different directions are given for the Jones apparatus (p. 1978).

Only the soda lime specified by the manufacturer of the basal apparatus should be used.

An extra tank of oxygen should be available. All the labels on the tank should be read to make certain that oxygen is being used. Other gases, such as nitrous oxide, have been sent to laboratories by mistake, with serious consequences.

Chapter 109

Apparatus for measuring basal metabolic rate

STANLEY REITMAN

COLLINS (BENEDICT-ROTH) APPARATUS

Description of the apparatus

This apparatus makes use of a water-sealed spirometer. One-way circulation, directing all exhaled air through the soda lime, is maintained by low resistance flutter valves. A large kymograph drum permits duplicate or even triplicate tests to be recorded on the same graph. It is also possible to extend the test period to as long as 15 min. if desired. The drum is driven by a precision spring-driven timing movement; a synchronous electric motor is optional.

Operating instructions

Put a chart around the kymograph drum, attach a sterile mouthpiece to the breathing tube, and fill the pen with ink.

Turn the 2-way valve handle so that the mouthpiece is in communication with room air and not with the contents of the spirometer.

Admit oxygen into the apparatus until the pen comes within 2 in. of the bottom of the paper, on the first vertical time line.

Admit the patient and allow him to relax on a comfortable couch or table for at least 30 min. before beginning the test.

Adjust the flexible arm of the apparatus so that the rubber mouthpiece comes near the patient's mouth and explain that the mouthpiece should be inserted so that the flange is between the gums and the lips and the 2 tabs are between the upper and lower teeth.

Apply the noseclip so that the sponge-rubber pads are well down on the nostrils, press them together firmly but gently, and adjust the thumbscrew to hold the noseclip in this position. Ask the patient to try to exhale slightly through the nose. If he can do this, the noseclip is not fitted properly and must be readjusted.

Turn the valve handle in its other position, which connects the patient to the breathing circuit of the machine. The oxygen bell and pen will now move up and down with each exhalation and inhalation.

At any convenient time set the clock in motion by moving its starting lever.

Note the thermometer and barometer readings.

Allow the test to run about 3 min., and as the pen crosses the third time line, place the leak tester weight on top of the bell and allow it to remain there until the pen crosses the next time line; then remove it and allow the test to continue another 3 or 4 min. If a leak is present, the test weight on top of the bell will cause the oxygen to escape, and the graph for the patient's respirations will assume a steeper angle. If there is evidence of leakage, stop the test and readjust the noseclip, mouthpiece, and tubing connections. Put more oxygen in the bell if necessary and proceed with the test, being certain to repeat the test for leakage again after 3 min.

After 7 or 8 min. end the test by turning the free-breathing valve back to the room air connection.

Shut off the clock and remove the mouthpiece and noseclip.

Note the thermometer and barometer readings. If there is a difference from those taken at the beginning of the test, take an average of the 2.

A duplicate test should always be performed, and it may be recorded on the same graph by starting the test 3 or 4 min. away from the beginning of the previous test.

If there is a leak of oxygen from the bell or any of the connections, the graph on the kymograph chart will rise rapidly.

If the patient is breathing outside air and not the oxygen in the tank, the line made by the pen will be almost horizontal.

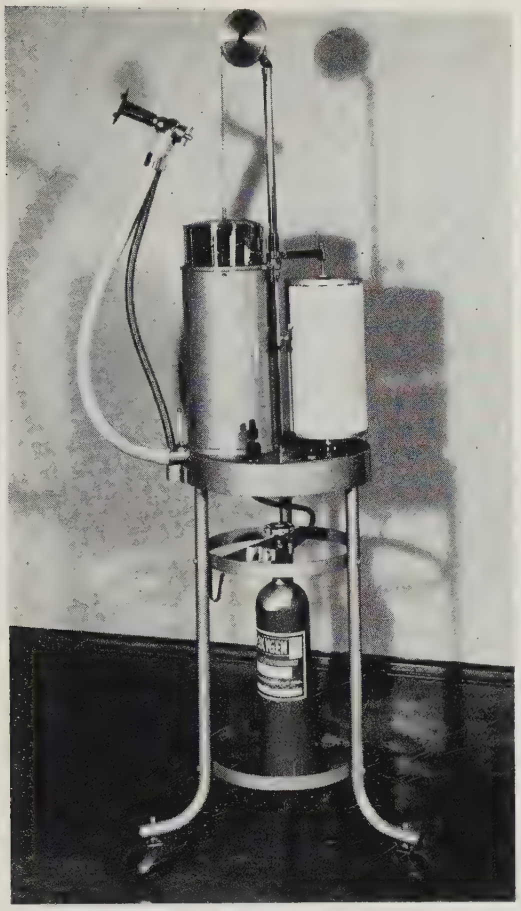

Fig. 697. Collins (Benedict-Roth) basal metabolism apparatus. (Courtesy Warren E. Collins, Inc., Boston, Mass.)

Obtain the patient's age, sex, weight without clothing, and height without shoes.

Calculation

Remove the chart from the drum and lay it flat on a desk. Using a ruler, draw a line through a majority, or an average, of the lower points. Note the rise in millimeters of this line in a 6 min. period on the chart. For example, if one end of the line cuts the vertical line at 47 mm. and 6 min. later it has risen to 121 mm., then the rise is 121 − 47 = 74.

To determine the normal*: Use Table "A" (Fig. 699). Rule a line to connect the "patient's height" found in column I with the "patient's weight" found in column II. The point at which this line intersects column III is the body surface area. Record this in the computation space allotted to it.

Now, using Table "B," find the calories/sq.M./hr., by using the patient's age (nearest birthday) and sex and record in space for computations. Multiply figure in column III by figure in Table "B" to obtain the total normal calories/hr., the exact normal for a person of this height, weight, age, and sex.

To determine the actual test: Record the difference in height of the 6 min. test period from the kymograph tracing in the space called "O_2 line."

Record the temperature of the oxygen bell from its thermometer and the prevailing barometric pressure in mm. Obtain the correction factor for pressure and temperature from Table "C" (the intersection of the 2 columns representing the temperature and pressure for this test). Record this in the proper computation space.

Multiply the figure recorded opposite "O_2 line" by the figure recorded opposite Table "C" to obtain the total actual test calories/hr.

To find the basal metabolic rate: Use the normal and the actual consumption of oxygen/hr.

Subtract the smaller from the larger figure.

Divide the difference by the normal. Move the decimal 2 places to the right to obtain basal metabolic rate in %.

If the actual test is greater than the normal, report as plus; if less than the normal, report as minus.

SANBORN METABULATOR*

Preparation of patient

The patient is prepared in the usual manner. He must be completely at rest mentally and physically, and 14 hr. must have elapsed since his last meal. He should lie down for 30 min. immediately preceding the test.

Preparation of apparatus

With the patient comfortable and resting, wheel the metabolism tester to a convenient position beside the bed and plug its power cord into the AC outlet.

Place the chromium-plated leak tester in a glass of cold water and allow it to remain there while the patient is being prepared. Place a sterilized rubber mouthpiece on the mouthpiece connector. Open the Plexiglas window over the writing stylus. Turn the breathing valve to the "test" position, thus connecting the bellows with the outside air, and then collapse the bellows by pushing the bellows carriage toward the absorption chamber as far as it will go. With the bellows in this position, turn the patient valve back to "fill," thus closing off the bellows. Now admit oxygen by carefully turning the handwheel on the oxygen tank to the left. Continue filling until the writing stylus is about 1 in. from the top edge of the paper; then close the valve on the oxygen tank tightly by turning it to the right.

Adjusting noseclip

The instrument is now ready for the test. Adjust the noseclip before inserting the mouthpiece. The sponge-rubber pads are used to apply pressure

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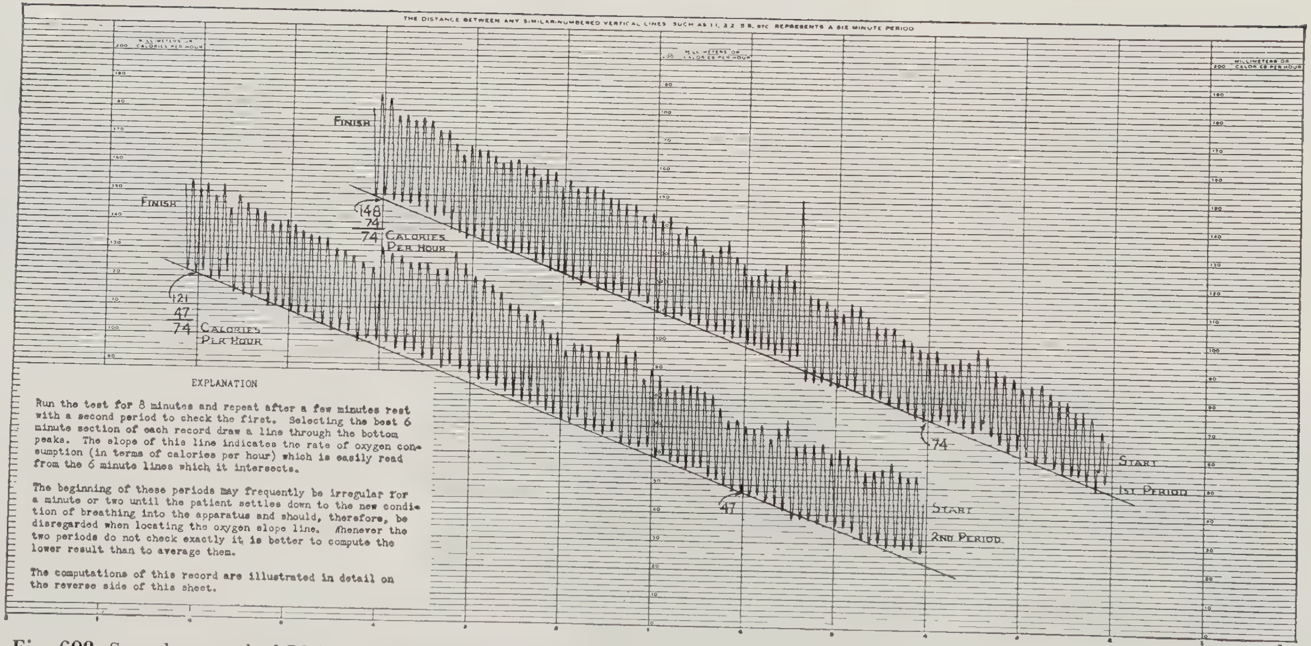
*These calculations are compiled from data furnished by Warren E. Collins, Inc., Boston, Mass.

*These data are compiled from facts furnished by the Sanborn Co., Cambridge, Mass. For further information refer to the brochure supplied with each Metabulator.



EXPLANATION

Run the test for 8 minutes and repeat after a few minutes rest with a second period to check the first. Selecting the best 6 minute section of each record draw a line through the bottom peaks. The slope of this line indicates the rate of oxygen consumption (in terms of calories per hour) which is easily read from the 6 minute lines which it intersects.

The beginning of these periods may frequently be irregular for a minute or two until the patient settles down to the new condition of breathing into the apparatus and should, therefore, be disregarded when locating the oxygen slope line. Whenever the two periods do not check exactly it is better to compute the lower result than to average them.

The computations of this record are illustrated in detail on the reverse side of this sheet.

Fig. 698. Sample record of BMR measurement using the Collins (Benedict-Roth) apparatus. (Courtesy Warren E. Collins, Inc., Boston, Mass.)

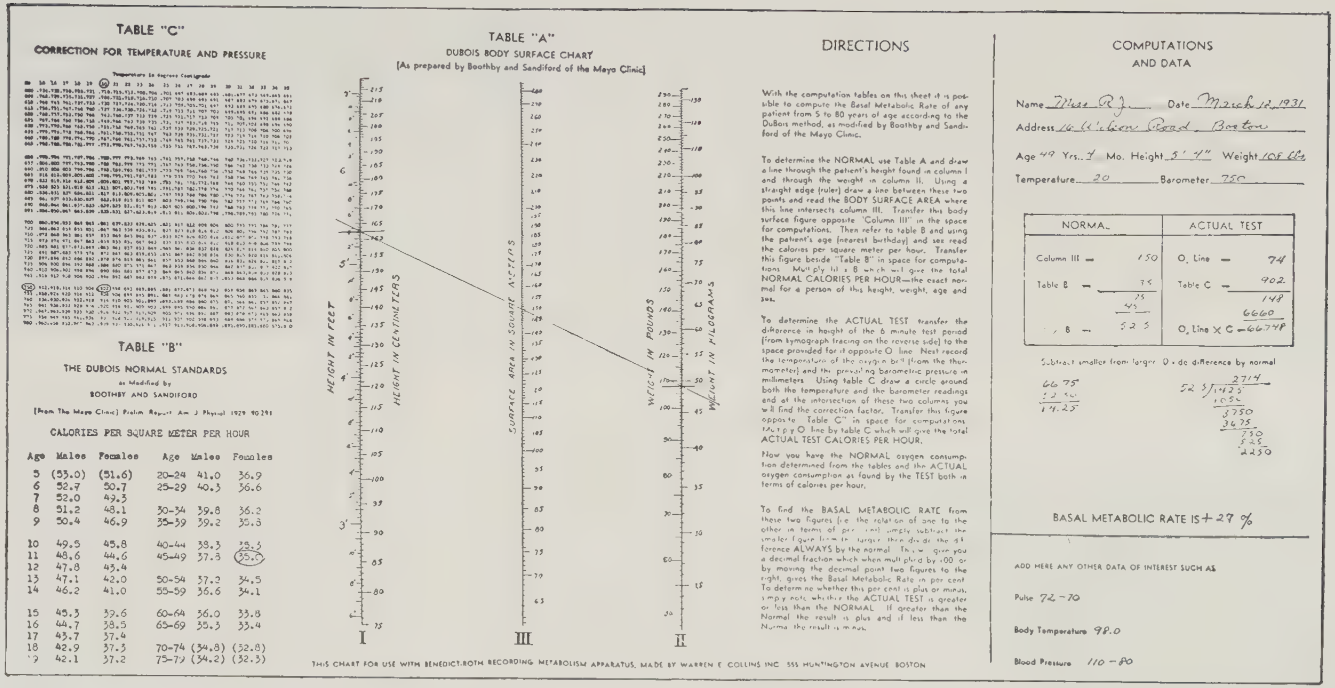


TABLE "C"

CORRECTION FOR TEMPERATURE AND PRESSURE

Temperature in degrees Centigrade

TABLE "B"

THE DUBOIS NORMAL STANDARDS

as Modified by

BOOTHBY AND SANDIFORD

[From The Mayo Clinic] Prelim Report Am J Physiol 1929 90 291

CALORIES PER SQUARE METER PER HOUR

Age	Males	Females	Age	Males	Females
5	(53.0)	(51.6)	20–24	41.0	36.9
6	52.7	50.7	25–29	40.3	36.6
7	52.0	49.3			
8	51.2	48.1	30–34	39.8	36.2
9	50.4	46.9	35–39	39.2	35.8
10	49.5	45.8	40–44	38.3	35.5
11	48.6	44.6	45–49	37.8	35.0
12	47.8	43.4			
13	47.1	42.0	50–54	37.2	34.5
14	46.2	41.0	55–59	36.6	34.1
15	45.3	39.6	60–64	36.0	33.8
16	44.7	38.5	65–69	35.3	33.4
17	43.7	37.4			
18	42.9	37.3	70–74	(34.8)	(32.8)
19	42.1	37.2	75–79	(34.2)	(32.3)

TABLE "A"

DUBOIS BODY SURFACE CHART

[As prepared by Boothby and Sandiford of the Mayo Clinic]

DIRECTIONS

With the computation tables on this sheet it is possible to compute the Basal Metabolic Rate of any patient from 5 to 80 years of age according to the DuBois method, as modified by Boothby and Sandiford of the Mayo Clinic.

To determine the NORMAL use Table A and draw a line through the patient's height found in column I and through the weight in column II. Using a straight edge (ruler) draw a line between these two points and read the BODY SURFACE AREA where this line intersects column III. Transfer this body surface figure opposite "Column III" in the space for computations. Then refer to table B and using the patient's age (nearest birthday) and sex read the calories per square meter per hour. Transfer this figure beside "Table B" in space for computations. Multiply III x B which will give the total NORMAL CALORIES PER HOUR—the exact normal for a person of this height, weight, age and sex.

To determine the ACTUAL TEST transfer the difference in height of the 6 minute test period (from kymograph tracing on the reverse side) to the space provided for it opposite O line. Next record the temperature of the oxygen bell (from the thermometer) and the prevailing barometric pressure in millimeters. Using table C draw a circle around both the temperature and the barometer readings and at the intersection of these two columns you will find the correction factor. Transfer this figure opposite "Table C" in space for computations. Multiply O line by table C which will give the total ACTUAL TEST CALORIES PER HOUR.

Now you have the NORMAL oxygen consumption determined from the tables and the ACTUAL oxygen consumption as found by the TEST both in terms of calories per hour.

To find the BASAL METABOLIC RATE from these two figures (i.e. the relation of one to the other in terms of per cent) simply subtract the smaller figure from the larger then divide the difference ALWAYS by the normal. This will give you a decimal fraction which when multiplied by 100 or by moving the decimal point two figures to the right, gives the Basal Metabolic Rate in per cent. To determine whether this per cent is plus or minus, simply note whether the ACTUAL TEST is greater or less than the NORMAL. If greater than the Normal the result is plus and if less than the Normal the result is minus.

THIS CHART FOR USE WITH BENEDICT-ROTH RECORDING METABOLISM APPARATUS, MADE BY WARREN E COLLINS INC 555 HUNTINGTON AVENUE BOSTON

COMPUTATIONS AND DATA

Name *Miss R. J.* Date *March 12, 1931*

Address *16 Wilson Road, Boston*

Age *49* Yrs. *4* Mo. Height *5' 4"* Weight *108 lbs*

Temperature *20* Barometer *750*

NORMAL	ACTUAL TEST
Column III — *1 50*	O₂ Line — *74*
Table B — *35*	Table C — *902*
75 *45*	*148* *6660*
[illegible] B — *52 5*	O₂ Line × C — *66.748*

Subtract smaller from larger Divide difference by normal

66 75
52 50
14.25

52 5)1425(.2714
1050
3750
3675
750
525
2250

BASAL METABOLIC RATE IS *+27 %*

ADD HERE ANY OTHER DATA OF INTEREST SUCH AS

Pulse *72–70*

Body Temperature *98.0*

Blood Pressure *110–80*

Fig. 699. Information necessary for calculating BMR from "raw" data. Table "C" is given here only to illustrate the method of obtaining the correction for temperature and pressure. This table will be found on the reverse side of all Collins kymograph sheets. (Courtesy Warren E. Collins, Inc., Boston, Mass.)

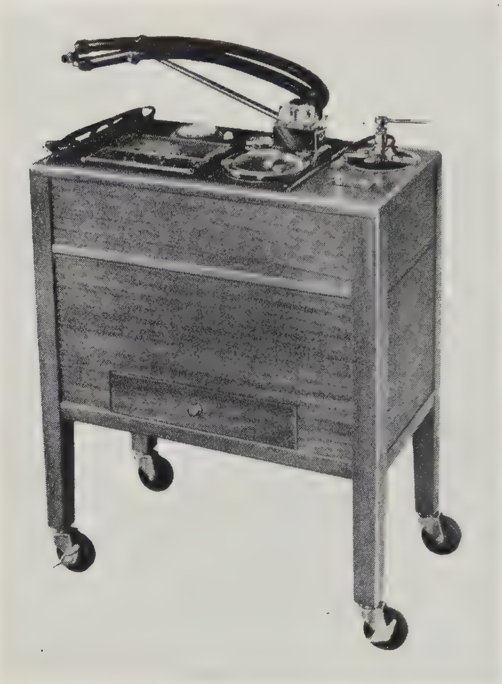


Fig. 700. Sanborn basal metabolism apparatus. (Courtesy Sanborn Co., Cambridge, Mass.)

against the soft sides of the nostrils. Loosen the thumbscrew so that the clip will fit loosely when first applied and then take the noseclip between the thumb and finger of both hands and, placing it over the nose near the bridge, slide it downward parallel to the patient's face until the pads are at the right level. Now tighten the thumbscrew until the patient finds he can no longer breathe out through his nose. Make sure of this by asking him to close his mouth and try. The noseclip must not be uncomfortably tight.

Placing mouthpiece

Adjust the position of the tester and of its flexible arm until the rubber mouthpiece is just in front of the patient's mouth, with its long axis pointing across the face. Insert the mouthpiece so that the 2 small projections lie between the upper and lower teeth while the flange of the mouthpiece lies between the lips and the teeth. See that the lips are not folded under, for this may permit leakage.

Dentures: Dentures will not cause trouble, especially if they fit well. Since there may be an air passage over the plate, however, it is doubly important with patients wearing artificial teeth to see that the lips make a good seal over the outside of the rubber mouthpiece. It is a mistake to remove the dentures for this may allow the mouthpiece to fall inward, away from the lips.

With patients with few or no front teeth, the problem is likely to become more difficult. This problem is solved, however, by having a good seal made by the lips. It may be necessary to draw the lips over the flange of the mouthpiece and hold them in place with adhesive tape.

Ruptured eardrums: If the patient has a ruptured eardrum and a patent auditory (eustachian) tube, air is likely to escape through the ear. If this occurs, the external ear canal is sealed with a cotton swab impregnated with petrolatum. The operator may have to hold the plug in place while the patient is being tested for leaks at the nose, for otherwise the plug may be blown out.

Making test

With the patient ready and breathing room air comfortably, start the record by turning on the motor switch. The stylus should now be tracing a horizontal line near the top of the record. The actual test is started by flipping the breathing valve to the "test" position, preferably at the end of an expiration. As soon as it is clear that the test is proceeding normally, check the seals at the patient's nose and mouth (and ears also, if necessary) with the leak tester. To do this, take it out of its glass of water, wipe it dry, and then hold it under the nose and at the corners of the mouth while watching for any condensation on the polished surface of the metal. The test should not be allowed to proceed unless all leaks have been eliminated, for a leak will spoil the accuracy of the oxygen-consumption measurement.

Run the test 10 min. or more unless the bellows become collapsed earlier. If a duplicate test is desired, turn the breathing valve to the "fill" position at the end of the first test, readmit oxygen from the tank as before, and when the bellows has been adequately filled, close the valve on the tank and return the breathing valve to the "test" position. If the patient shows any signs of discomfort or reluctance at the end of the first test, however, remove the mouthpiece and noseclip and allow him to rest before making a second run.

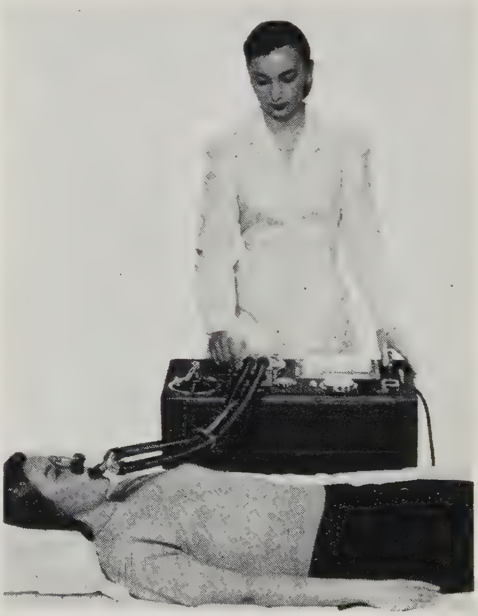


Fig. 701. Basal metabolism test using the Sanborn instrument. (Courtesy Sanborn Co., Cambridge, Mass.)

At the beginning of each test record the **temperature,** as read from the instrument's dial thermometer, and the **barometric pressure,** directly on the record. Tap the barometer gently before making the reading.

With the test completed, throw the breathing valve to the "fill" position again and remove the mouthpiece and noseclip from the patient. Pull carefully on the protruding paper in order to bring the remainder of the record out of the machine; then bend the paper sharply back over the barometer dial and move the free end a little to one side, whereupon a quick pull will tear off the protruding portion of the paper.

Be sure the motor switch is turned off. Write the date and the patient's name, height, weight, age, and sex on the record and also the temperature and barometer readings if this has not already been done.

Measurement

Since the volume occupied by a given amount of gas depends on the temperature and pressure, the statement that a patient's oxygen consumption is so many ml./min. means little unless the conditions under which the volume measurement was made are specified. However, temperature and pressure are likely to be different in each test, so that it has become accepted practice to refer all gas-volume measurements to "standard temperature and pressure," or "STP" for short. Standard temperature is 0° C. or 32° F., standard pressure is 1 atmosphere or 760 mm. of mercury. The actual volume measurement is made under the conditions prevailing during the experiment, and then the operator calculates, using the gas laws, the volume that **would have been found** if the measurement had been made under STP conditions. This is what is meant by an oxygen-consumption rate as "x ml. per minute, STP."

A measuring scale has been provided that eliminates the STP calculation.

The purpose of this test is to determine the patient's rate of oxygen consumption expressed in ml./min. at standard temperature and pressure. This is done by estimating the **average downward trend** of the patient's respiratory tracing. To do this draw a "slope line" through the top peaks of the record. The top peaks are chosen because they represent the expiratory position of the patient's lungs, which is usually a little more regular than the inspiratory position. The slope line can be drawn through the top, bottom, or the middle of the record as long as it represents the average downward trend. If the record is regular, the slope line can easily be drawn through the top peaks. If the record is jagged, the operator must exercise some judgment in deciding what represents the average slope. If the respiratory tracing is very irregular, it is useless to attempt to place a slope line, and the test must be repeated.

Lay the record on a flat surface and with a long ruler and a dry pen, pencil, or other scratching device draw a straight slope line to represent the average downward trend of the tracing. Extend this slope line to the edges of the record.

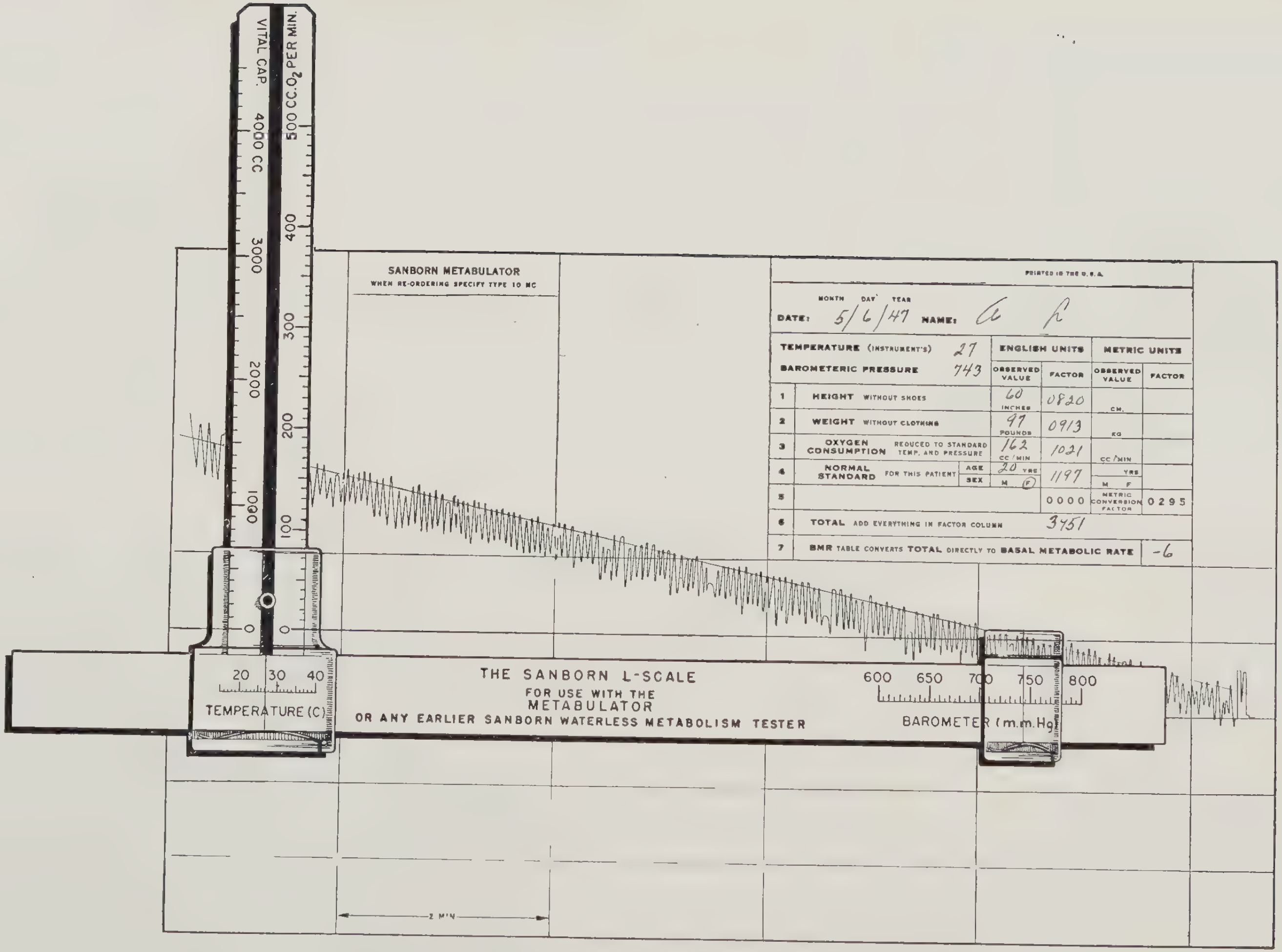
L-scale: A measuring device has been designed to enable the operator to read the oxygen consumption in ml. at STP/min. directly from the record. The L-scale will be found among the accessories in the case with the "slide-rule calculator." Hold the long arm of the "L" horizontal, with the lettering right-side up, and slide the shorter arm (pointing upward) over the left-hand end of the long one. The small slider will already be in place at the right-hand end of the arm. The scale now consists of a horizontal bar carrying 2 sliders, each with a vertical hairline lying over a correction scale (Fig. 702). One of these scales is for temperature and the other for barometric pressure. Adjust the sliders so that the hairlines mark the temperature and barometer readings taken during the test. Note that each slider also carries a **horizontal** hairline, lying a little above the upper edge of the long arm of the "L." The line on the right-hand slider has a vertical hairline crossing it, and the L-scale is to be applied to the respiratory tracing in such manner that the intersection of these 2 hairlines on the slider lies exactly over the intersection of the previously drawn slope line and one or other of the horizontal lines printed on the record (Fig. 702). When this has been done, adjust the "levelling" of the "L" until the horizontal hairline on the **left-hand** slider also coincides with the horizontal line that has been chosen. Check the position of the intersection on the right-hand slider again, to be sure that it has not moved, and then read the oxygen consumption directly at the point where the slope line strikes the right-hand margin of the vertical arm of the "L." The reading on this latter scale is the patient's oxygen consumption in ml./min., reduced to standard temperature and pressure.

T-scale: While the L-scale represents a considerable improvement over the T-scale that has been supplied with the Sanborn waterless metabolism tester, it is perfectly permissible for any operator who wishes to do so to use the T-scale on the records produced by the present machine. It is applied to the tracing exactly as in the case of the waterless testers, using a vertical line for the alignment and reading the oxygen consumption along the horizontal arm. Corrections for the temperature and pressure are made, of course, by adjustment of the sliders before the scale is applied.

Conversely, the L-scale may be used to measure a slope line produced on one of the older waterless machines. Since alignment of the L-scale depends on a **horizontal** line, it will be necessary to draw in a base line on the waterless chart, which can be done most conveniently before the chart is removed from the machine. Simply inflate the bellows to the point where the writing stylus will cross the respiratory record near its end and then, maintaining the bellows at this volume, give the drum one full turn by hand. This will provide a horizontal base line for the L-scale. If it has been placed correctly, as suggested above, the base line and the slope line will cross near the right-hand edge of the record. If the base line is too high or too low, it can easily be drawn in a more appropriately placed line parallel to the original.

Basal metabolic rate

The Metabulator will provide the operator with a measurement of the patient's oxygen consumption in STP ml./min. The BMR goes a step further—it is a measure of how nearly normal the de-


SANBORN METABULATOR
WHEN RE-ORDERING SPECIFY TYPE 10 MC
PRINTED IN THE U. S. A.
VITAL CAP.
4000 CC
3000
2000
1000
0
500 CC.O_2 PER MIN.
400
300
200
100
0
THE SANBORN L-SCALE
FOR USE WITH THE
METABULATOR
OR ANY EARLIER SANBORN WATERLESS METABOLISM TESTER
20
30
40
TEMPERATURE (C)
600
650
700
750
800
BAROMETER (m.m.Hg)
2 M'N

DATE: MONTH DAY YEAR 5/6/47 NAME: a l

	TEMPERATURE (INSTRUMENT'S) 27	ENGLISH UNITS		METRIC UNITS	
	BAROMETERIC PRESSURE 743	OBSERVED VALUE	FACTOR	OBSERVED VALUE	FACTOR
1	HEIGHT WITHOUT SHOES	60 INCHES	0820	CM.	
2	WEIGHT WITHOUT CLOTHING	97 POUNDS	0913	KG	
3	OXYGEN CONSUMPTION REDUCED TO STANDARD TEMP. AND PRESSURE	162 CC/MIN	1021	CC/MIN	
4	NORMAL STANDARD FOR THIS PATIENT AGE	20 YRS	1197	YRS	
	SEX	M [✓ F]		M F	
5			0000	METRIC CONVERSION FACTOR	0295
6	TOTAL ADD EVERYTHING IN FACTOR COLUMN		3451		
7	BMR TABLE CONVERTS TOTAL DIRECTLY TO BASAL METABOLIC RATE				-6

Fig. 702. Sanborn "L-scale" for use with the Metabulator. (Courtesy Sanborn Co., Cambridge, Mass.)

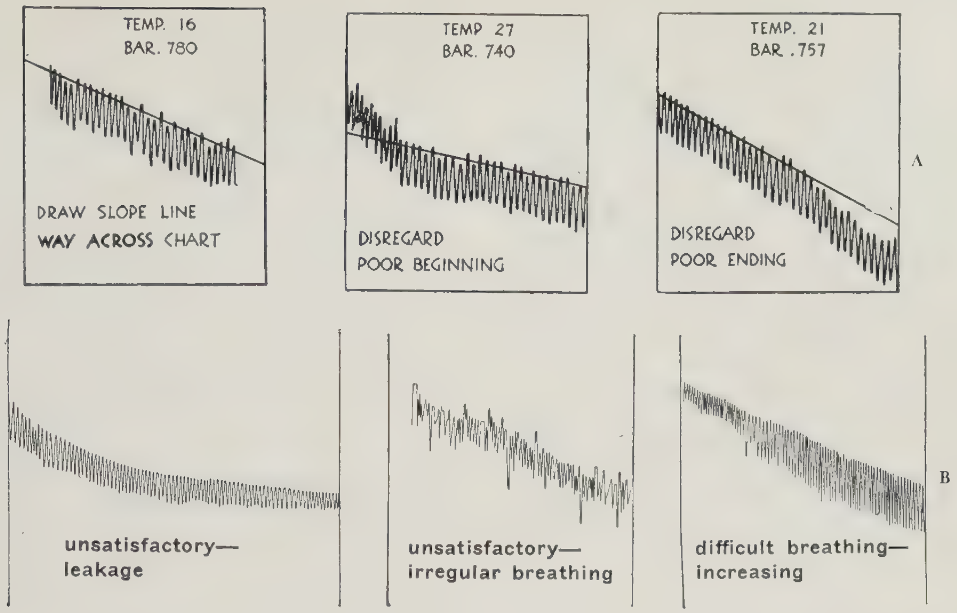


Fig. 703. A, How to draw slope lines, Sanborn basal metabolism test. **B,** Types of slopes that are to be discarded in making Sanborn basal metabolism tests. (Courtesy Sanborn Co., Cambridge, Mass.)

termined oxygen consumption is for the particular patient concerned, taking his height, weight, age, and sex into account. The BMR is expressed as a figure (with a prefixed plus or minus sign) showing the **percentage** by which the patient's oxygen consumption is above or below normal. Thus a BMR of +42 indicates an oxygen consumption 42% too high; while −17 indicates one 17% below normal, etc. The problem now is how to convert the figure found for oxygen consumption into a BMR for the patient concerned, thus telling by how many percentage points the oxygen consumption differs from the normal.

This conversion may be accomplished either by the "Slide-Rule Calculator" supplied with the accessories or by using metabolism tables.

Slide rule calculator

Adjust the hairline to oxygen consumption (corrected to standard temperature and pressure).

Shift the slides to bring the height under the hairline.

Reset the hairline to actual present age, male or female.

Move the slider to bring the weight under the hairline.

Read basal metabolic rate over the BMR arrow.

Metabolic tables

The tables formerly used with Sanborn metabolism testers are perfectly adequate to convert oxygen consumption to BMR. The new set of tables will greatly facilitate the calculation involved. The printing on the recording paper is designed so that the whole computation can be carried out on the original record.

The quantities concerned in the calculations are (1) the patient's height, (2) his weight, (3) his rate of oxygen consumption (L-scale reading), and (4) his age and sex.

The newer tables give a factor for each of these 4 measurements, and a column is provided on the chart for the factors found, so that they can be put down one above the other. They are then added together, and the sum is converted directly to a BMR through the use of a final conversion table.

English or metric units: The principle involved in this method of calculation makes it possible to use the same tables whether the heights and weights are in pounds and inches on the one hand or in kilograms and centimeters on the other. The difference is taken care of by the addition of a "metric conversion factor" if the metric units are used. Examine the printed box on one of the charts. The tabulations are set up so that if the bodily measurements are taken in English units the left-hand section is used, with no conversion factor; whereas if the measurements are in centimeters and kilograms the right-hand column is used and the metric conversion factor is printed at the bottom of the factor column so that it will be added in along with the other factors. The sum in line 6, obtained by the addition of all the figures in the factor column, will be the same whether the left-hand section with English units is used, with no conversion factor to add in, or whether the right-hand section with metric units is used and the conversion factor "0295" added in. Since the sum in line 6 is the same for either system of units, the BMR in line 7 will also be the same, as it should be.

Height factor: Measure the patient's height to the nearest inch or centimeter. In the column at the left-hand side of Table 1 on the recording paper will be found the heights arranged in steps of 10. Suppose the patient's height is 67 in. Since 67 = 60 + 7, follow the left-hand column down to "60," then follow that horizontal line across to the column headed "+7." This factor, 0750, is the correct one for a height of 67 in. Other values will be treated in exactly similar fashion. If the height is 182 cm., follow the height column down to "180" and then follow the horizontal line across to the column headed "+2," where the factor 0121 will be found, etc.

Weight factor: Measure the patient's weight without clothing in pounds or kilograms.* Find the weight factor in Table 2 on the recording paper. For example, if the patient's weight is 145 lb., follow the weight column down to "140" and then trace that horizontal line over to the "+5" column, where the factor 0765 will be found.

Oxygen-consumption factor: Use Table 3 on the recording paper to find the factor for oxygen consumption. The procedure is the same as for the other 2. The figure to start with is the number of STP ml. of oxygen/min. as determined from measurements of the patient's record. For a consumption rate of 217 ml./min., for example, find "210" in the left-hand column and then follow that line across to the column headed "+7." The factor is 1275.

Normal standard factor: Since the basal metabolic rate depends on a comparison of what the patient actually does with what a normal person of his age and sex ought to be doing, a factor must be found representing "normal" behavior. Table 4, showing the factors for the normal standard, will be found on the back of the tables card. The column for the age is in the middle, and the factors for male patients are found at the left, whereas those for females are to the right. Some of the factors are the same for a number of different ages. This is all right, for that is the way normal people behave. The groupings also differ between males and females.

Find the patient's "normal standard" factor and pull it down in line 4; e.g., for a female patient 29 years of age it would be 1208. For a male of the same age it would be 1114.

Conversion to BMR: When all the factors have been added (including the metric conversion factor if the height and weight were in centimeters and kilograms) the total is converted directly to BMR through the use of Table 5 on the recording paper. Reduce the sum to 3 figures by discarding the fourth, increasing the third figure by 1 if the fourth is 5 or more. Thus, if the total is 4316, call it "432"; whereas if it is 4312, it becomes "431." Using the conversion table just as the others were used, it will be seen that "431" gives a BMR of +43. Insert the result in line 7 on the chart.

JONES METHOD

This method was devised by Dr. Horry Jones of Chicago. The machine is a portable instrument, consisting of a bellows mounted on a strong metallic stand with a standard liter measuring device that automatically corrects its oxygen volume for atmospheric pressure and temperature.

The metabolism test is a quantitative determination and therefore requires more than ordinary precision in technic. Results should be within 1-5% of the patient's true metabolic rate; otherwise the technic is being very crudely performed.

Set up the apparatus as illustrated. Carry out each step in the exact sequence as given.

Connect the oxygen tank in place using a wheel wrench to tighten it against the leather gasket. Place the tracing sheet on the clock plate with the lettered end of the clock plate up.

Adjust clock plate so that its geared bar fits in the slot of clock housing and then slide the plate down into the slot until it comes to rest against the geared wheel inside of housing. If the clock starts, stop it by snapping the switch to OFF position. If it does not start with the switch in ON position, lift the clock plate about ½ in. and bring it down with slight downward jerk. Then see if the clock can now be started by turning on the switch. If this has succeeded in getting the geared bar and geared wheel to mesh, stop the clock until it is to be started as directed later.

Keep the pen tip free of dried ink or lint by drawing a pinpoint through it so that the pen will write with a fine, sharp line. Fasten the pen to its bar and rotate the clock in its seat until the pen traces evenly across the entire width of the clock plate. Now attach the mouthpiece (disinfected, not by boiling but by briefly immersing into wide-mouthed jar of 50% alcohol, and then rinsed with water) to the breathing tubes and rotate so that

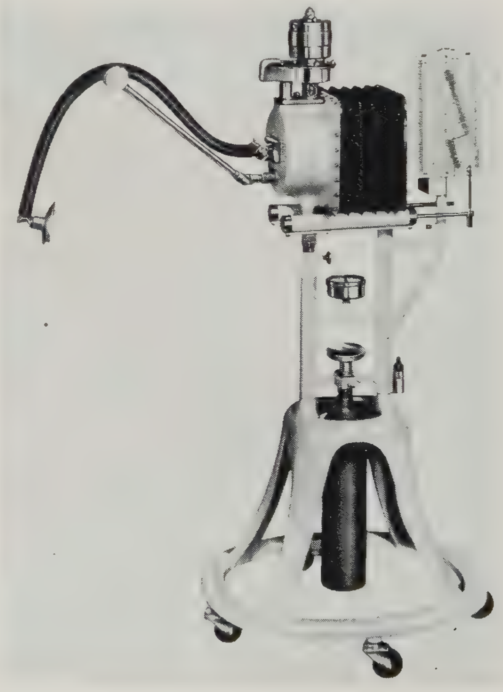


Fig. 704. Jones basal metabolism apparatus. (Courtesy Jones Metabolism Equipment Co., Chicago, Ill.)

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*Needless to say, heights and weights must be either **both** English or **both** metric; do not try to mix a metric height with an English weight or vice versa.

it faces the patient's mouth squarely without twisting the tubes.

Stand the apparatus at exactly the correct distance (move apparatus to patient, not the patient to the apparatus) so that the mouthpiece will just reach the patient's mouth without bending the breathing tube.

Turn the 3-way valve so that its pin is parallel to the floor. Pull on the bellows until the point of the pen is at the air position on the graph. Holding the bellows in that position, turn the 3-way valve perpendicular to the floor, thereby holding the pen point at the air position.

Close the needle valve. This retains the oxygen in the measuring chamber.

Using the wheel wrench, admit oxygen into the measuring chamber until the black indicator is ½ to ¾ of the distance to the red indicator. Shut the oxygen tank valve.

Open the needle valve and admit enough of this oxygen into the bellows to push the pen point to S, M, or L on the paper (small, medium, or large, depending on the size of the patient).

Admit an exact liter of oxygen into the measureing chamber by turning the wheel wrench on the oxygen tank until the black needle is superimposed on the red needle of the gauge.

Take the patient's pulse and record.

Apply the nosepiece to the patient. Connect the mouthpiece to the apparatus. Instruct the patient how to use the mouthpiece and insert it into the patient's mouth.

To get the patient to relax at the beginning of the test and to overcome the tendency to erratic breathing, have the patient take one deep, forced full inspiration, then exhale slowly, and relax the arms and shoulder muscles for normal, even breathing.

Start the motor of the apparatus.

At the end of the patient's expiration turn the 3-way valve parallel to the floor, thus connecting the patient to the bellows.

After a few respirations start the clock and make the pen write on the graph.

When the tracing has crossed the base line at A (receded within the black line on the paper), open the needle valve and admit the known liter of oxygen from the measuring chamber into the bellows. This will push the pen out beyond the black line. When the pen recedes within the black line again at B, the test is ended.

Take the patient's pulse and record.

Repeat the test; the preliminary test alone is never sufficient.

Testing for nose leak

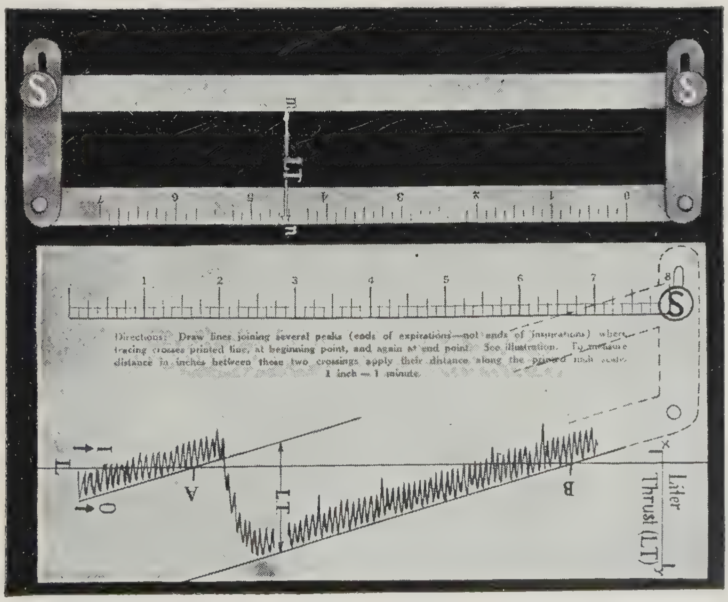
After the noseclip has been placed in position instruct the patient to try to breathe through his nose. If he is able to breathe through his nose while the clip is in place, adjust it to make it airtight. If there is a nose leak, the tracing will be concave instead of a straight line slope.

Testing for mouth leaks

If there is a mouth leak due to improper adjustment of the mouthpiece, the tracing will be a back-and-forth movement of the pen instead of a slope.

Calculation of basal metabolic rate

Use the protractor to determine the time required to consume 1 L. oxygen, as follows: Apply the top edge of the top ruler of the adjusted protractor along the long or "liter" slope of the tracing on the paper to line up with the highest peaks. Do not allow more than 1 or 2 peaks to project above the ruler edge. Holding the upper rule in this



Directions: Draw lines joining several peaks (ends of expirations—not ends of inspirations) where tracing crosses printed line, at beginning point, and again at end point. See illustration. To measure distance in inches between these two crossings apply their distance along the printed inch scale.
1 inch = 1 minute.

Fig. 705. Jones basal metabolism protractor and sample test chart. (Courtesy Jones Metabolism Equipment Co., Chicago, Ill.)

position, swing the connecting arms to such position that either edge of the connecting arm lies PARALLEL to the edge of either end of the tracing sheet. The shorter slope peaks should now line up with the upper edge of the lower ruler. If they do not, it indicates (1) that a full liter was not admitted to the breathing chamber, (2) that part of it was lost through a nose leak or mouth leak, (3) that soda lime has been used for more than 100 tests, or (4) that the patient was not made to relax for even breathing by taking a full, deep inspiration.

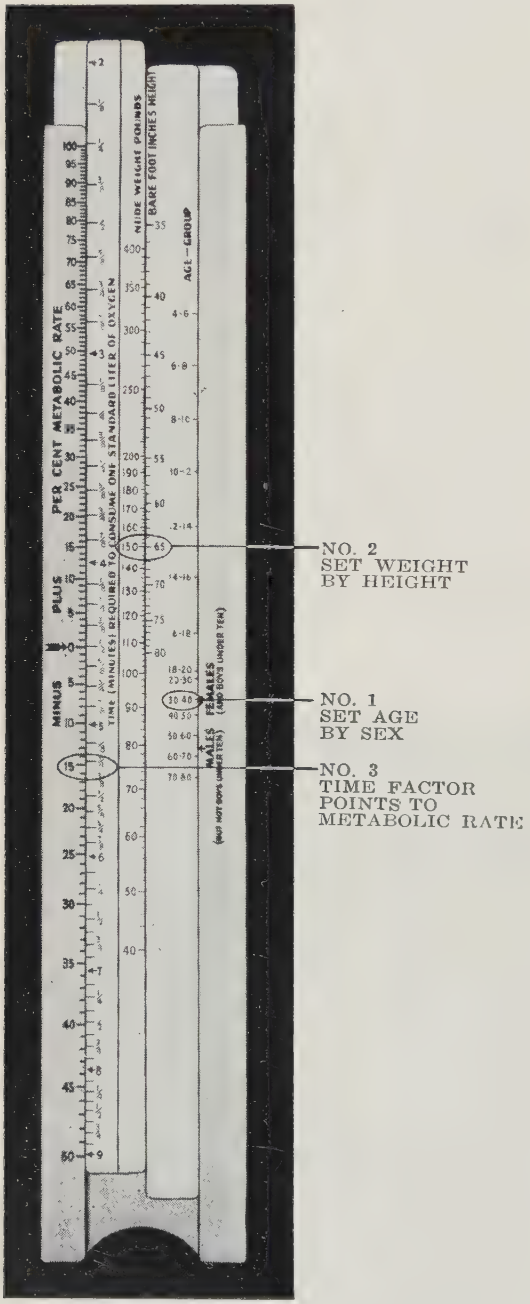


Fig. 706. Jones basal metabolism calculator. (Courtesy Jones Metabolism Equipment Co., Chicago, Ill.)

Draw a line along the top of the 2 edges of the protractor. A is the point at which the shorter or first slope crosses the printed line; B is the crossing of the longer slope. The distance between them, in inches, is the time in minutes required by the patient to consume the standard liter of oxygen.

Obtain the age of the patient, his weight without clothing, height without shoes, and sex.

Read off the basal metabolism rate on the automatic calculator (Fig. 706).

Determination of liter thrust of pen

When a liter of oxygen is discharged into the bellows, the pen moves outward by the amount of the expansion of the bellows. This is called the liter thrust of the pen. Measure a liter of oxygen in the usual way. Do not have the apparatus connected to a patient for this part of the procedure.

Next open the 3-way valve and pull the bellows carried by the hand to a point such that the pen tip is about ¾ in. inside the printed line of the tracing sheet; then close 3-way valve to hold the bellows at that point. Let the clock plate run to trace a short line (X) to show this position of the pen tip. Stop the clock plate.

Discharge the liter of oxygen now in the measuring chamber, making sure the indicator swings from the room temperature point on the gauge scale backward all the way to within ⅛ in. of the self-verifying zero of the gauge. The self-verifying zero is a short, black calibration mark somewhere near and slightly above the word "caution" at the edge of the gauge dial. If the indicator does not make this full swing, a liter of oxygen was not delivered to the bellows.

After all of the oxygen has gone into the bellows, i.e., after the pen has been thrust out as far as this liter pushes it, again let the clock run to trace another short line (Y) to show its new position. The distance between (X) and (Y) is the "liter thrust."

This part of the procedure should be done independently of the test, i.e., any time after or before the test. It is done only for the purpose of adjusting the rulers of the protractors and occasionally as a checkup to see if the protractor rulers are still adjusted to equal the distance of the liter thrust.

To adjust the rulers of the protractor to fit this liter thrust, release the knurled nuts of the short arms of the protractor; then move the rulers closer in or farther apart until the upper edge of one ruler coincides with (X) and the upper edge of the other ruler coincides with (Y). Adjust both ends of the protractor so that the upper edges of rulers equal the distance from (X) to (Y) throughout the entire length of the protractor; then tighten the knurled nuts to hold the rulers to that adjustment. At sea level and 25° C. this liter thrust of the pen is approximately $1\frac{7}{16}$ in., and for each 1000 ft. elevation above sea level this liter thrust is increased about $\frac{1}{16}$ in. For example: At about 6000 ft. above sea level the liter thrust would be $1\frac{7}{16}$ in. plus $\frac{6}{16}$, or $1\frac{13}{16}$ in. This serves as a means of determining whether or not the standard liter is actually delivered regardless of the barometric pressure changes caused by changes in elevation above sea level.

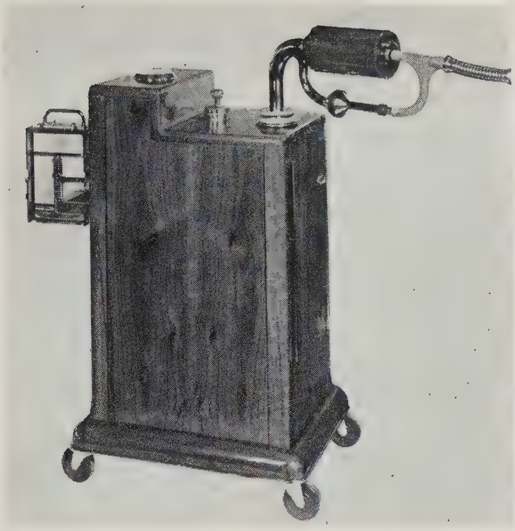


Fig. 707. Jones air basal metabolism instrument. (Courtesy Jones Metabolism Equipment Co., Chicago, Ill.)

Having determined the liter thrust for the sea-level elevation of the locality, this thrust is approximately constant, and thereafter it is unnecessary to adjust the rules of the protractor to equal the liter thrust, except as an occasional checkup.

JONES DOUBLE BELLOWS MOTOR-BASAL

This apparatus differs from the Jones motor-basal in that there are 2 bellows instead of 1 (one inside the other). The larger of the 2 bellows has exactly twice the cross-section area of the smaller bellows.

By means of a conversion valve a single turn converts the apparatus to either a small bellows or a large bellows hookup.

The smaller bellows hookup is used on a small-sized patient, who would be connected to the apparatus for an unnecessarily long period if a large capacity bellows were used. The large bellows hookup is used on a large-sized patient, who would be connected to the apparatus a relatively short time if a small bellows were used. Thus the capacity of the apparatus is adjustable to the size of the patient.

JONES AIR BASAL METABOLISM INSTRUMENT

The Jones air basal metabolism instrument utilizes air instead of oxygen in determining the BMR. Different volumes (800-2285 ml.) are obtainable, permitting the examiner to chose the volume best suited for the particular patient. Two timer speeds are also available.

Operating instructions

Take the patient's pulse and temperature and record.

Sweep out the used air of the previous test by taking hold of the sweep-out stopper and lifting the air-sweep up and down with about 2 or 3 full strokes of moderate speed, each up and each down stroke taking about 2 sec.

Adjust the elevator assembly to the correct height so that the mouthpiece is in the proper position for insertion.

Select the volume connection to be used in the preliminary test by referring to the table "For Preliminary Test" on the reverse of the slide rule calculator. Insert the volume pin in the hole in the T-bar corresponding to the volume selected. The push button on the side of the recording instrument should be at the IN position (1 in./min.) for this preliminary test.

Rest the sweep-out stopper on the rim of the sweep-out opening (in center of cabinet top).

Apply the nosepiece, making certain that there is no leak.

Insert the mouthpiece. The patient has access to free breathing through the sweep-out opening. To get patient to relax at the beginning of the test and to overcome the tendency to erratic breathing, have the patient take one deep, forced, full inspiration and then exhale and relax the arms and shoulder muscles for normal, even breathing.

Take hold of the knob at the top edge of the bellows panel and pull the panel out as far as it will come. While holding the bellows panel out with one hand, insert the sweep-out stopper in position with the other hand; then release the bellows panel. (In high altitudes, 4000 ft. or more, it may prove desirable to insert the sweep-out stopper at the beginning of expiration.)

Push the sweep-out stopper in a slightly slanted position to permit slow leakage of air past the stopper, while the outermost swing of the rocker stylus slowly recedes to the desired beginning point of the tracing sheet, which is about 3-4 lines farther out than the lines corresponding to the line or space pointed at by the VoluMeter indicator; then straighten the sweep-out stopper to gas-tight position, pressing down slightly.

Wait about 1 min.; then bring the rocker stylus and the T-bar stylus against the paper by turning the knurled knob for each stylus so as to bring the tip of each in contact with the paper.

Start the timer by operating the switch on top of it. Observe that the timer plate is actually descending. Continue the test until the graph completely crosses the midline.

Stop the timer, release each stylus, and remove the test chart. Remove the mouthpiece; then remove the nosepiece after checking to make certain a leak did not develop during the test.

To use the graph

Refer to the reverse side of the slide rule calculator for directions for determining "minutes per liter."

Using the table "For Repeat Tests"' decide whether a better volume connection could be used for a repeat test.

If examination of the graph reveals irregular breathing, a repeat test should be performed in which the volume is doubled, and the timer speed is halved (by pulling out the button on the timer).

At least one repeat test must always be made; the preliminary test alone is never sufficient.

Take and record the patient's pulse.

To prepare for the next test, take hold of the sweep-out stopper and lift the air-sweep up and down with about 2 or 3 full strokes of moderate speed, with each stroke taking about 2 sec.

If the apparatus is to be used again immediately, rest the sweep-out stopper on the rim of the sweep-out opening and proceed again as above. If the apparatus is not to be used immediately, leave the sweep-out stopper resting on the rim of the sweep-out opening as a means of hanging the sweep-out rod. This permits the air chamber to be ventilated.

Calculations

After determining the "minutes per liter," determine the BMR by reference to the opposite face of the slide rule calculator.

Section Two

ELECTROCARDIOGRAPHY

JULIUS ELSON

The frequency with which disorders of the cardiovascular system are encountered in the practice of medicine and the importance of the electrocardiograph in the diagnosis, treatment, and prognosis of these disturbances have made it desirable to include a discussion of the important subject of electrocardiography in this book.

It is not within the scope of this section to undertake an explanation of the theories underlying electrocardiographic interpretation and all the patterns that present themselves in clinical practice. These subjects are covered thoroughly in standard texts devoted solely to the subject of electrocardiography. This section will present the principle and the use of the electrocardiograph and will point out the more important patterns, clinical applications, and pitfalls of electrocardiography.

USE OF ELECTROCARDIOGRAPH

Because the terms **electrocardiograph** and **electrocardiogram** are so often confused, it is well to point out that **electrocardiograph** refers to the recording instrument, whereas **electrocardiogram** refers to the record itself. The electrocardiograph is an instrument by means of which a visible record is made of the many minute electrical or electrochemical changes occurring in the **heart muscle** associated with its contraction. It is important to stress the point that the electrocardiogram is a reflection merely of electrical activity of heart **muscle** and not dynamic activity so that one will recognize the limitations of the clinical application of this means of diagnosis.

For instance, in valvular disease or in certain congenital malformations in which the heart muscle is not primarily involved, the electrocardiogram cannot be expected to be of help except in those cases in which secondary heart muscle changes occur, usually in the form of hypertrophy of the atrial or ventricular musculature. These secondary effects on the musculature give a clue to the primary valvular disease; but until these effects do occur the electrocardiogram is often normal in the presence of valvular disease.

The electrocardiogram may be normal even in the presence of myocardial involvement if the site of the lesion is not in the proper axis with regard to the electrocardiographic "leads."

All laboratory tests must be interpreted in the light of the clinical picture. Laboratory tests alone should rarely if ever be relied upon for making a diagnosis or determining treatment and prognosis. This is an extremely important point and must constantly be borne in mind. In the experience of many electrocardiographers, patients are often referred for electrocardiographic tracings in the belief that such tracings may give the answer to the whole question of diagnosis. Actually, so many factors can affect electrical action currents in the heart that even experienced electrocardiographers may not be able to make proper electrocardiographic diagnoses without ample clinical data. Even with this information it may be impossible to make a decision in borderline electrocardiograms because of the many variations of the normal electrocardiogram.

Whereas a normal electrocardiogram does not necessarily exclude the presence of cardiac disease, an abnormal tracing surely indicates some disorder. This disorder may be organic in nature or it may involve disturbances in the cardiac mechanism; or it may merely reflect an abnormal psychosomatic effect on the heart. Many extracardiac influences can alter the electrocardiogram. Toxic substances, either bacterial or nonbacterial, and disturbances in other organs which might affect the position of the heart in the thoracic cavity are among the factors that sometimes alter the tracing. For example, drinking cold water, emotional disturbances, and smoking may change the normal electrocardiographic pattern.

VALUE OF THE ELECTROCARDIOGRAPH

The greatest value of the electrocardiograph lies in the field of coronary artery disease in which the myocardium is involved acutely or by progressive degenerative changes. The electrocardiograph in these conditions is of great value since in many instances there are no other objective positive clinical findings.

Often in acute myocardial infarction the electrocardiogram will present a pathognomonic picture, especially if serial electrocardiograms are taken. These electrocardiograms are found to undergo characteristic changing patterns. Although acute myocardial infarction without significant electrocardiographic abnormality does occur, such a diagnosis should be made with reservation.* The diagnosis can often be made clinically, but it is a great comfort to the clinician to have the added assurance of the electrocardiographic diagnosis. This may be of vital importance to the patient also, since on occasion the symptoms may be confusing and may simulate other conditions such as an acute surgical abdomen or dissecting aneurysm. Patients have been operated upon for perforated peptic ulcer when in reality they were suffering from myocardial infarction; others have been spared needless surgery by making an electrocardiogram that disclosed the cardiac nature of the patient's symptoms.

In addition to its value in coronary artery heart disease, electrocardiography is very useful in the positive recognition of certain cardiac arrhythmias and disturbances of conduction, the exact nature of which could not be ascertained without recourse to cumbersome methods of recording venous pressures, arterial pulses, and the apex impulse. The electrocardiograph has made the certain identification of these abnormal mechanisms a simple matter.

This instrument can sometimes be of aid as a guide to treatment. Recognition of characteristic alterations of certain segments of the tracings or of clinically obscure disturbances in rhythm and conduction that have occurred in the course of treatment with certain drugs, most commonly digitalis, is often the signal for discontinuation or more cautious administration of such drugs.

Recognition of the effects of electrolyte disturbances on the electrocardiogram, chiefly those of increases or decreases of potassium, has enabled the electrocardiograph to be a useful tool in the recognition and management of such disorders.

From a prognostic standpoint, conclusions from an electrocardiogram alone are most precarious. Prognosis should be made from an evaluation of the clinical aspects of the case. For instance, the presence or absence of heart failure, which is not indicated by the electrocardiogram, is very important. A patient with myocardial infarction may show an electrocardiogram approaching a normal pattern, yet he may die of cardiac rupture, embolism, ventricular fibrillation, or heart failure.

The characteristic alterations of the electrocardiogram caused by digitalis have led to another field of usefulness for the electrocardiograph, i.e., in the standardization of digitalis glycosides, using human subjects.

PRINCIPLE OF ELECTROCARDIOGRAPHY

The science of electrocardiography was first made possible in the middle of the nineteenth century, when it was discovered that the contraction of the frog's heart was accompanied by an electric current. By connecting the outside surface of the human subject by means of electrodes to a capillary electrometer, Ludwig and Waller in 1887 demonstrated that the electrical currents in the heart could be recorded. Because of the nature of the instrument, records at that time were inaccurate, and the method was not practicable. It was not until 1903, when Einthoven applied his string galvanometer to the study of the human heart, that electrocardiography was put on a firm, clinically reliable basis. Only with an understanding of the electrocardiogram in electrical terms can one appreciate the magnitude of Einthoven's contribution to medicine.

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*Elevation of serum glutamic oxalacetic transaminase activity is frequently important corroborative evidence in such instances (p. 129).

The electrocardiogram, as recorded by electrodes placed upon the body surface, consists of a series of impulses, each of which is of relatively short duration. The baseline above and below which these impulses fluctuate is known as the **isoelectric level.** Some of these impulses may rise from the isoelectric level to their peak in a time interval as short as 0.02 sec. while the amplitude may represent as little as 1 mv.

Furthermore, the resistance of the circuit within the patient, the **patient circuit** as measured between a pair of electrodes, averages under good conditions about 2000 ohms. On the basis of 1 mv. amplitude and 2000 ohms resistance offered by the patient, the peak power that the body generating the electrical impulses can deliver to the recording instrument would be only one-eighth of one-thousandth of one-millionth of a watt.

String galvanometer: The string galvanometer is based upon the fundamental principle that a conductor that carries an electric current and is suspended in a magnetic field will tend to be deflected from its original position by a force acting on that conductor. This force is produced by the reaction between the current and the magnetic field.

String shadow: Until a few years ago the type of electrocardiograph solely used was the "string" type, in which the current-carrying conductor is a quartz "string" about $\frac{1}{5000}$ in. in diameter (5μ), covered with a metal such as platinum. This string is suspended between the north and south poles of an electromagnet. Without stretching this platinum-coated quartz string very tightly one may time this light fiber to a sufficiently high natural frequency of vibration to produce a moving system capable of following the details of the human electrocardiogram with a satisfactory degree of accuracy.

The actual motion of the "string" through which the current from the patient passes is so small that it must be magnified 500-1000 times. This is accomplished by means of a microscope through which light is thrown by an electrical source and condenser. The magnified shadow of the "string," with all its movements, is thus projected on a camera. The recording medium, usually bromide paper, is pulled through the camera at a constant speed. A timing device is provided by a spoked wheel rotating in the path of light and periodically interrupting the light to produce timing coordinates, thus allowing measurement of the various events of the cardiac cycle.

With most timers using a rotating wheel, the fifth spoke is wider, so that every fifth vertical time line is broader than the other 4. The distance between each fine line represents 0.04 sec.; that between each wide line, therefore, represents 0.2 sec.

A piece of glass with parallel lines ruled on it is mounted just in front of the paper so that it casts fine horizontal shadows on the sensitized paper at 1 mm. intervals. The resulting horizontal lines are the coordinates by means of which the amplitude of the electrical deviations is measured.

This combination of the string shadow, the light interruptions produced by the timer, and the ruled scale for the amplitude coordinates results in a completed record in which the string movements and the time and amplitude coordinates appear as white lines on a dark background.

Leads: There are 3 main types of leads in general use today: (1) the bipolar or conventional leads, (2) the unipolar leads, and (3) the augmented unipolar leads. These leads are applicable no matter what type of electrocardiograph is used.

Bipolar leads: These are obtained by placing metal electrodes on 2 different points of the body. By convention, and because experience has justified it, these leads are recorded from the right arm, left arm, and left leg, as well as from various positions on the chest.

The lead taken from the right and the left arm is known as **Lead I;** that from the right arm and the left leg is called **Lead II;** and that from the left arm and left leg is called **Lead III.** These are the conventional bipolar leads universally used.

The chest leads are designated in a variety of ways, depending on the position of the chest electrode or **exploring electrode** and the limb electrode or **indifferent electrode.** For further clarification of certain complexes of the electrocardiogram even esophageal leads have been used; in these a specially designed electrode is passed down into the esophagus and electrocardiograms are taken with this electrode held at various levels.

In recent years the method of unipolar lead electrocardiography has been widely adopted. These leads more nearly record the absolute potential at each of the extremities as well as at the exploring electrode on the chest wall.

Unipolar leads: There are 2 types of unipolar leads: (1) the Wilson type and (2) the generally adopted augmented unipolar limb leads. Unipolar leads are described on p. 1993.

The use of "unipolar" limb and chest leads has become general. The connections are made as for the "central terminal" (V) leads of Wilson, described on p. 1996. Three records are then made, with the precordial electrode placed on the right arm, on the left arm, and on the left leg, respectively. A discussion of the theoretical considerations and clinical applications of the unipolar leads can be found in *Unipolar lead electrocardiography* by Emanuel Goldberger[1] and many other publications devoted to this subject.

The choice of the lead or electrode pair to be recorded is made by a **lead selector** switch on the panel of the instrument.

Standard records: Records made at different times or by different instruments, in order to be comparable, must record the same electrical quantity according to the same scale. The unit of measurement is the millivolt, and the scale of recording is 1 cm., which corresponds to 1 mv. developed at the electrodes.

To standardize an electrocardiograph the sensitivity of the recording device is so adjusted that it responds to a 1 mv. current by deviating 1 cm. from the base line or zero level. The 1 mv. standardizing current is introduced from a source built into the instrument.

Before standardizing a string galvanometer type of electrocardiograph it is necessary that the patient, the galvanometer, and the standardizing circuit all be connected. Resistance in the patient circuit varies from time to time and from lead to lead; for this reason it is necessary to restandardize the instrument for each lead while the string and patient circuit are connected and the string is responding to cardiac action currents. This is not necessary in the modern direct writing electrocardiograph.

Skin resistance: The patient circuit resistance derived from the skin must be kept low, 2000 ohms or less.

Skin resistance is lowered by the application of some form of electrode paste or jelly to the skin at the site of the electrode. This paste contains an electrolyte; when rubbed into the skin with a little excess on the electrode, it reduces the resistance between the electrode and the tissues beneath the skin from a value of many thousands of ohms to about 1000 ohms.

Electron tube amplifier: Another type of electrocardiograph was later developed and is now in common use. It employs the electron tube amplifier principle. This type of instrument makes use of an amplifying circuit which greatly magnifies the tiny cardiac currents and includes a very large resistance to prevent overshooting. One such type instrument employs a very small mirror attached to the movable element or coil in the magnetic field. This mirror is so arranged that by its motion it swings either a spot of light or a field of light with a shadow in it across the face of the recording camera.

An advantage of this type of apparatus is that it is not necessary to standardize the instrument with the patient switched in. This enables the operator to make the tracing without any disturbance to the recording beam other than that caused by its response to the standardizing pulse. Still another advantage is that standardization need not be repeated after the patient is switched in, nor for each of the leads, as in the case with the string electrocardiograph. These advantages are possible because of the relatively high resistance and current in the amplifying circuit as compared with the slight resistance caused by the skin or by polarization. All of these electrocardiographs require the use of photographic paper or film for recording the electrocardiogram.

Direct writing electrocardiographs: Direct writing electrocardiographs have in most places supplanted the photographic types of instruments. These apparatus, by use of high-power radio tube amplifiers, have made possible direct, immediately visible recordings of the electrocardiogram. In addition to this very real advantage, the need for photographic materials and a dark room is obviated.

VECTORCARDIOGRAPHY

This method of electrocardiographic study is not new, but in the past few years it has been studied with greatly renewed interest and emphasis. There are 2 ways of analyzing electrocardiograms. One is the conventional or "pattern" method, with which we are largely concerned in this chapter, and the other is the "vector" method of analysis. In the former we learn by experience and correlation with pathologic specimens the various electrocardiographic patterns associated with disease entities, whereas in the latter the many electrical forces developing and receding in the cardiac cells are represented by an arrow, the length of which measures their resultant magnitude and the tip, their direction at any given instant. These arrows, representing the magnitude, direction, and sense of this force at a given instant, will vary from moment to moment as the impulses travel through the heart fibers in the various directions. These "instantaneous vectors" can be constructed from the conventional electrocardiogram taken to include the 3 bipolar limb leads, the 3 unipolar limb leads, and the 6 unipolar chest leads. By connecting the tip of each arrow representing the vector of electrical potential from moment to moment, a "loop" is formed, graphically depicting the direction of the vectors throughout the cardiac cycle. These vector loops can be much more accurately depicted by means of an oscillograph connected with the body by means of specially placed electrodes.

This method of interpretation has the advantage of not requiring memorizing of the numerous patterns with their wide range of normality; one merely visualizes abnormalities in direction and magnitude in the electrical potentials.

The **spatial vectorcardiogram** has shed light on a number of electrocardiographic problems in which the information derived from the electrocardiogram was obscure.

It has helped in the diagnosis of left ventricular hypertrophy in which the spatial vectorcardiogram usually has a typical appearance. The electrocardiogram in many cases of left ventricular hypertrophy may be normal or have alterations of doubtful significance.

In the detection of myocardial infarction in the presence of left bundle branch block, the spatial vectorcardiogram is quite useful since the electrocardiographic diagnosis of myocardial infarction is usually impossible in this situation.

In some cases of left ventricular hypertrophy, the QRS complex may be unusually prolonged to 0.12 sec. or more, creating confusion as to whether left bundle branch block may be present. The spatial vectorcardiogram will help in the differential diagnosis.

In many cases of left ventricular hypertrophy, diffuse myocardial damage may be present without evidence from the electrocardiogram. The spatial vectorcardiogram may give such a clue.

In myocardial infarction that is old or healed or in cases in which the electrocardiographic tracing is atypical, the diagnosis may be very difficult. The vectorcardiogram may be useful in such cases.

There are serious disadvantages to the routine adoption of spatial vectorcardiography as a routine method of study in clinical practice. The chief of these perhaps is the difficulty at this date of determining time relationships of the various phases of the cardiac cycle and the inability to identify arrhythmias accurately. Vectorcardiography should still be regarded as an experimental method, and until the objections mentioned above are obviated and until some standard method of lead placement is generally agreed upon, it perhaps should not be adopted as a routine method in clinical practice.

Chapter 110

Use of the electrocardiograph

JULIUS ELSON

Detailed descriptions of all the electrocardiographs on the market and their step-by-step operation will not be given since they vary in certain respects from each other. Complete information can be found in the brochures that each manufacturer supplies with the instrument. Because direct writing instruments have largely supplanted electrocardiographs that employ photographic methods, only the operation of a typical commonly used direct writing electrocardiograph will be described.

All electrocardiographs are delivered in proper working condition and are accompanied by a pamphlet giving complete detailed instructions for operating the instrument. Since maintenance and care of the instrument vary with the type of apparatus used, it is best to refer to these pamphlets for such information. In choosing an electrocardiograph it is advisable to bear in mind the availability of service for it.

VISO-CARDIETTE, MODEL 100*

One of the most commonly used types of direct writing instrument is the Viso-Cardiette. The Viso-Cardiette produces an electrocardiogram or graphic record of the electrical waves (action potentials) generated by the beating of a patient's heart. The record reads from left to right to represent the passage of time, the standard speed being 25 mm./sec. The paper is ruled in 1 mm. squares. The light vertical lines on the graph mark off 1/25 sec., or 0.04 sec., per square, and the heavy lines 1/5 sec. (0.2 sec.).

The vertical deflections of the recorded tracing show the electrical changes taking place in the heart. Each mm. square between the lighter horizontal lines represents 1/10 mv.; each square within the heavier horizontal lines represents ½ mv. The short lines above the graph printing indicate 3 sec. intervals.

Complete operating instructions, including checking the shipment and the instrument, accompany the instrument on purchase.

Operation of the Viso-Cardiette, Model 100

The Viso-Cardiette must not be operated on direct current or on alternating current having a frequency different from that marked on the serial name plate. Although 50-cycle current will not damage a 60-cycle instrument, it will make the recording paper run at a speed equal to 5/6 of the rated value. Direct current will blow the fuse in the instrument.

Remove all watches from the patients and the operator unless they are of the nonmagnetic variety. Watches may be easily demagnetized by gradual withdrawal from the center of an aircore coil carrying alternating current.

Zero temperatures will cause no damage if the Viso is warmed before operation by turning the power switch to ON for ½ hr. or more. Avoid high temperatures, such as are caused by direct sunlight. Avoid knocks and jars when carrying.

Preparation of instrument: The Viso-Cardiette should be placed on a table or other firm flat support, with the 2 cover latches facing the operator. Pull down on the top of both latches to release and swing back the cover. See that the power switch Off-On-Run is turned to OFF. Open the transparent cover and read the power requirements (volts and cycles) stamped on the name plate, visible below the hinge at the right. See that the electric power matches these requirements. Do not use any Viso on a direct current power line or on a 25-cycle power line.

Be sure that there is sufficient Permapaper in the Viso; then close the transparent cover.

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*This section is abstracted from a pamphlet furnished by the Sanborn Co., Waltham, Mass., and is published with their permission.

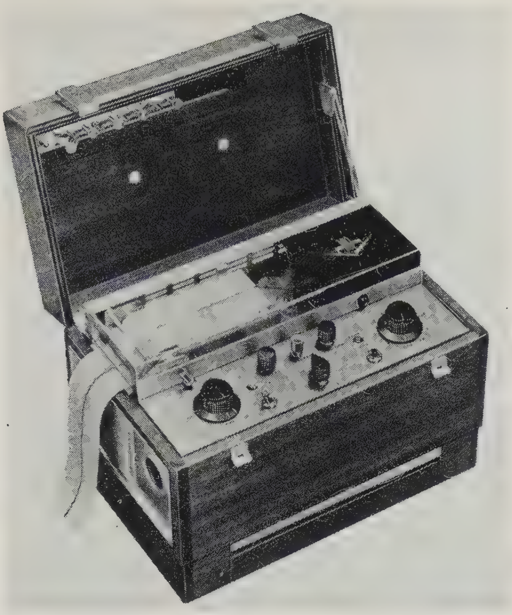

Fig. 708. Sanborn Viso-Cardiette, model 100. (Courtesy Sanborn Co., Waltham, Mass.)

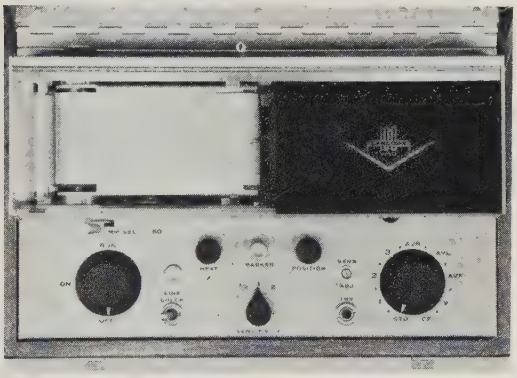

Fig. 709. Top view, Sanborn Viso-Cardiette, model 100. (Courtesy Sanborn Co., Waltham, Mass.)

Remove the power cord from the pocket in the case cover and plug the female end of the cord into the Viso power receptacle on the left side of the cabinet; plug the other end of the cord into a wall socket or other power outlet.

Turn the lead selector switch, at the extreme right side of the panel, to the small dot between STD and 1.

Turn the power switch Off-On-Run, at the extreme left side of the panel, to ON. The red indicator will light.

Let the apparatus warm for at least 2 min.

Turn the position control back and forth and watch the writing arm. It will be near the top of the Permapaper when the control is to the right (clockwise) and near the bottom of the paper when the control is to the left (counterclockwise).

Move the writing arm by means of the position control to the center of the paper and leave it in this position.

Turn the lead selector switch to STD.

With one hand clear of the apparatus, use one finger to touch the top of the Line Check button, which is directly above the Line Check switch, to see if touching this button will make the writing arm vibrate. Then throw the Line Check switch to the other position and touch the top of the Line Check button again to see if the writing arm will vibrate. Leave the Line Check switch in the position where the writing arm will **not** vibrate when the Line Check button is touched.

To test the paper drive, turn the power switch Off-On-Run back and forth between ON and RUN a few times. The paper will feed out of the apparatus while the switch is at RUN.

With the switch at RUN, move the speed shift lever 25 mm./sec.–50 mm./sec., between 25 and 50 a few times. The paper speed will be 25 mm./sec. while the lever is at 25 and 50 mm./sec. while the lever is at 50.

Turn the power switch back to ON. Leave the speed shift lever at 25.

To test the stylus heat, turn the power switch to RUN. Turn the stylus heat control clockwise until the stylus is a dull red and then turn fully counterclockwise. Watch the base line (black trace) formed by the hot stylus at the end of the writing arm. The base line will flood when the knob is to the right (clockwise) and the stylus is red hot. The base line will be less black and not flooded when the knob is to the left (counterclockwise) and the stylus has less heat.

Adjust the stylus heat controls for a firm, dense, black base line, with clear, well-defined edges. Once set, this setting is usually good for the 50 mm./sec. speed, but a slight adjustment may be necessary.

Turn the power switch back to ON.

To test the marker button, turn the power switch to RUN.

Press the marker button a few times, looking for the series of marks that appear in the margin of the Permapaper. Turn the power switch back to ON.

1 MV button:

Turn the Sensitivity switch to 1. Turn the power switch to RUN. Turn the lead selector switch to STD.

Press the 1 MV button and then release it. Repeat a few times. The base line will deflect each time, to form a standardization pulse on the paper. Turn the power switch back to ON.

Standardization:

Turn the power switch to RUN.

Turn the Sens Adj control back and forth with a screwdriver, furnished with the apparatus, while recording a series of standardization pulses with the 1 MV button. Notice the height of these pulses. The standardization pulses are largest when the Sens Adj control is to the right (clockwise) and are smallest when the Sens Adj control is to the left (counterclockwise).

Set the Sens Adj control for standardization pulses which are exactly 10 small divisions or 2 large divisions high.

Turn the Sensitivity switch to ½. Press the 1 MV button a few times. The standardization pulses will be 5 small divisions high.

Now turn the Sensitivity switch to 2. Press the

1 MV button a few times; the standardization pulses will be 20 small divisions high. Return the Sensitivity switch to 1.

Turn the power switch back to ON.

To turn off Viso:

Turn the Lead Selector switch to the small dot between STD and 1. Turn the power switch to OFF.

Unplug the power cord from the power outlet, unplug the other end from the Viso, coil it up, and replace in pocket of the case cover.

Check to see that the power cord and the patient cable are in the case cover pockets and that the accessories are neatly packed away in the Viso drawer and slide on the inside of the top cover; then close the case. Do not force the cover.

To prepare Viso for operation:

Combine all the preceding steps into one sequence, and practice this sequence until the preparation can be made without referring to the directions.

Open the Viso. Check the power required against the power available.

Set the power switch to OFF and connect the Viso to the power with the power cord.

Turn the lead selector switch to the dot between STD and 1. Turn the power switch to ON. Warm for 2 min. See that there is Permapaper in the Viso.

Turn the lead selector switch to STD. Perform the line check (writing arm does not vibrate) with the Line Check button and switch.

Center the writing arm on the paper with the position control.

Turn the power switch to RUN with the speed shift lever at 25. Adjust the base line density with the heat control.

Set the Sensitivity switch to 1. Record a standardization pulse, 1 MV switch, and a marking indication, marker button. Inspect the recording for base line quality, for a definite marking indication at the margin of the Permapaper, and for a standardization pulse which is exactly 10 small divisions high. The Sens Adj control should not have to be reset if it was properly set at the beginning.

Turn the lead selector switch to the dot between STD and 1.

Preparation of patient

Have the patient lie on an examining table or preferably a large comfortable cot or bed.

See that the patient is warm, relaxed, and not apprehensive. A few words of reassurance will be helpful.

Expose the patient's forearms and the calves of both legs.

It is most important that there be good contact between the skin and the electrode. This is obtained with Redux.* Press ½ in. of Redux from the tube onto a spot on the inner side of the patient's forearms, halfway between the wrist and the elbow. Do the same on the inner side of each calf.

Using a gauze pad or the corner of a cloth towel, or a tongue depressor, rub the Redux into the skin, covering an area of about 2 sq.in. Rub until a slight reddening of the skin is noticeable; 10 strokes usually suffice.

Connecting patient to apparatus

After rubbing, leave a slight moist residue of Redux.

Fasten the electrodes over this residue with the rubber straps. The strap hooks over the pin. Draw the strap tight enough for firm contact but not so tight as to cause discomfort.

Remove the patient cable from the pockets of the case cover.

Connect the ends of the patient cable leads to their corresponding electrodes, screwing them in place tightly with the thumbscrew.

RA lead (white)—fastens to the right arm electrode.

LA lead (black)—fastens to the left arm electrode.

RL lead (green) fastens to the right leg electrode.

LL lead (red)—fastens to the left leg electrode.

C lead (brown) is ignored for the present; coil it up, out of the way.

Turn the lead selector switch to the dot between STD and 1.

Plug the free end of the patient cable into the blue socket at the right end of the apparatus. The slot at the end of the patient cable plug mates with the projection inside the patient cable socket.

Disconnecting patient

See that the lead selector switch is set to the dot between STD and 1. Disconnect the patient cable from the socket.

Disconnect the ends of the patient cable leads from the electrodes.

Release the rubber straps and remove the electrodes.

With a damp towel sponge the Redux from the patient's arms and legs.

Wash the electrodes with soap and hot water. Rinse well. Do not use steel wool or any other abrasive.

Wipe any Redux from the rubber straps and the patient cable tips with a damp towel.

After drying, place the patient cable, rubber straps, and electrodes in their proper storage place and close the cover.

Taking the various leads

Lead I:

Prepare the apparatus and the patient.

Turn the lead selector switch to 1. The writing arm will flicker part way across the paper about once each second.

Turn the power switch to RUN. Press the marker button once briefly. Run a few inches of Permapaper through the Viso.

The recording should show a dense, black base line with clear, well-defined edges, a steady recording, which does not wander up and down on the paper, and a definite, unmistakable lead marking. If the record does not fulfill these requirements, more practice is needed before taking the electrocardiogram of a patient.

While recording the lead, insert a standardization pulse into the flat section of the electrocardiogram.

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*Redux is a special jelly made by the Sanborn Co., Waltham, Mass.

Leads II, III, aV_R, aV_L, aV_F: These are taken as Lead I, except that the lead selector switch is turned to the proper lead. Lead II is marked with 2 short lead marks, Lead III with 3 short lead marks, Lead aV_R with 1 long lead mark, Lead aV_L with 2 long lead marks, and Lead aV_F with 3 long lead marks. All leads must show standardization, which is accomplished, as explained above, by inserting a standardization pulse.

The tracing should show a dense black base line with clear, well-defined edges; a prominent standardization pulse inserted just after the T wave so that the standardization will appear on a flat section of the recording; a definite, unmistakable lead marking for each lead of the recording; and a steady recording that does not wander up and down on the paper.

Chest lead:

Prepare the Viso-Cardiette for operation.

Prepare the patient, attaching the 2 arm electrodes and the 2 leg electrodes.

Prepare the locations on the chest wall as shown in Fig. 710.

Apply Redux and rub until the skin reddens. Important: Do not allow the Redux at one chest position to contact the Redux at another chest position, since this would interfere with and spoil the electrocardiogram. Prepare only enough area with Redux at any one location to match the area of the electrode.

Snap the chest electrode handle onto the electrode post and fasten the chest electrode to the C (brown) patient cable tip. If preferred, the Welsh vacuum cup electrode may be used in place of the standard electrode and handle.

Grasp the end of the chest electrode handle. Hold the chest electrode against one prepared location on the chest wall.

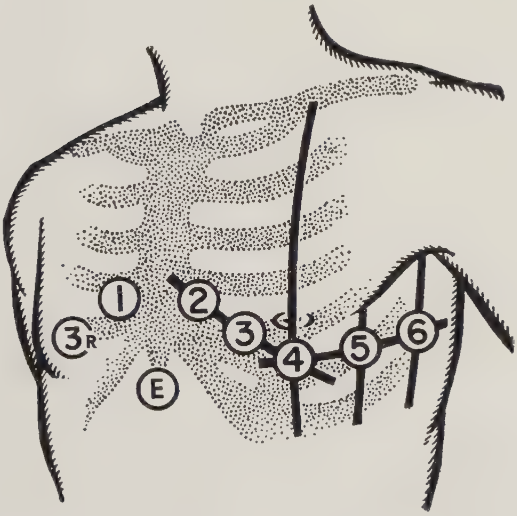

Fig. 710. Chest electrode standard positions for chest leads. **1,** Fourth intercostal space, at right border of sternum; **2,** same interspace, at left sternal border; **3,** midway between positions **2** and **4; 4,** fifth intercostal space, midclavicular line; **5,** same level as **4,** in anterior axillary line; **6,** same level as **4** and **5,** in midaxillary line. (Courtesy Sanborn Co., Waltham, Mass.)

Turn the lead selector to V, standardize, insert one extra-long lead mark, and record a few inches of the electrocardiogram.

To take the various chest leads, place the chest electrodes at the various sites as indicated in Fig. 710.

Replacement of Permapaper: Every roll of Permapaper has a star marked on it 30 ft. from the end as a warning that the paper is about to run out. Replace the paper as follows:

Turn the power switch to OFF. Lift the transparent cover at the rear of the Viso panel.

Press the lock lever to the right to unlock the writing table. This lever is located near the center of the transparent cover hinge.

Lift up the right end of the writing table and swing it back as far as it will go and then remove any unused paper. Clean any deposits from the writing plate at the end of the writing table with a damp cloth.

Mount the new roll of Permapaper on the spindle beneath the end of the writing table. The paper should unwind to the right, off the top of the roll, as the operator faces the apparatus.

Unroll about a foot of the paper and lay it back over the rubber brake roll.

Lift up the pressure roll at the left end of the writing table with the lift bar. Feed the end of the paper under the pressure roll and release the lift bar. Pull on the paper to take up the slack.

Be sure that the paper does not catch on the stylus while pressing the writing table back in place. Press the lock lever to the left.

Turn the power switch to RUN for a few moments to check for proper loading and to allow the paper to assume its proper tracking position.

Special electrocardiograms: Most electrocardiograms are recorded with the Sensitivity switch at 1. If the deflections of any recorded lead should be too large to fit conveniently on the Permapaper, set the Sensitivity switch to ½, press the 1 MV button, and then record the lead, which will then appear at ½ amplitude. If the recorded lead is too small for convenient viewing on the paper, set the Sensitivity switch to 2, press the 1 MV button, and then record the lead, which will then appear at twice the amplitude.

Bipolar chest leads: The CF bipolar chest lead is available by use of the lead selector switch.

The CR and CL bipolar chest leads are available by connecting the tip of the LL (left leg) lead wire to the chest electrode, located at its desired position on the chest. Then set the lead selector switch to lead 2 position for a CR recording, or set the lead selector switch to lead 3 position for a CL recording.

Maintenance, adjustments, and trouble shooting

Power fuse replacement: If the Viso will not operate at all, one or both of the power fuses may be blown. The 2 fuses are located behind the removable plate on the left-hand side of the Viso. To replace, remove the plate, turn the fuse knob ½ turn to the left, and pull out. Remove the blown fuse from the cap, replace with the new fuse, reinsert in place, and screw the fuse knob back in place; then replace the plate. The replacement is 0.6A slow blow fuse Sanborn No. 26 B-8.

Patient fuse replacement: If the Viso is inoperative at all lead positions (stylus goes off-scale, then drifts back on-scale 30 sec. later, with no EKG pulses, apparently normal on STD position), the patient fuse may need replacement. This fuse is located on the right-hand side of the Viso cabinet, next to the patient cable socket. To replace, turn the fuse knob ½ turn to the left and pull out. Remove the blown fuse, replace it with the new fuse, and screw the fuse knob back in place. The replacement fuse is 5 ma. fuse Sanborn No. 26A-1.

Indicator light replacement: If the Viso operates normally, but the red plastic lens will not light, the indicator lamp, visible upon lifting the transparent cover, will not light, this indicator will need replacing. To replace, lift the transparent cover, turn the lamp ½ turn to the left, insert the new lamp, press in firmly, and turn ½ turn to the right to fasten in place. The replacement lamp is Sanborn No. 30A-20.

Writing arm maintenance and replacement: The writing arm may need maintenance or replacement when a dense, sharply defined base line on both top and bottom of the tracing cannot be obtained at any position of the heat control. Turn off the apparatus, lift the transparent cover, and inspect the stylus ribbon to see if it is not broken or bent or tilted so that it is not flat against the paper. Check to see that the stylus writing surface is in contact with the paper.

If the stylus ribbon is not flat against the paper, loosen the writing arm clamping screw, tilt the writing arm to bring the stylus writing surface flat against the paper, and then tighten the clamping screw again.

If a satisfactory base line still cannot be obtained, loosen the writing arm clamping screw and lift out the writing arm. Inspect the writing surface of the stylus. If it is dirty, clean it by gently scraping it with a sharp instrument or by rubbing with No. 00 or finer sandpaper. Never use steel wool. Replace the writing arm and tighten the clamping screw.

Turn on the Viso and check again for a dense, sharply defined base line at some position of the heat control. If the base line is still not satisfactory, tighten the stylus pressure adjusting screw by turning clockwise until the stylus barely clears the Permapaper. Then slowly loosen the adjusting screw by turning counterclockwise for a satisfactory tracing, which should occur within the first full rotation of the screw.

If a satisfactory base line still cannot be obtained, return the stylus pressure adjusting screw to its original setting and loosen the clamping screw that holds the stylus in place. Remove the defective writing arm and tighten a new writing arm in its place.

Somatic tremor: Somatic tremor is identified by a fuzzy, totally irregular base line. It is usually caused by a tense or uncomfortable patient. Check the patient's comfort and reassure him. See that the electrode straps are not too tight.

Alternating current interference ("AC"): Alternating current interference is identified by regular sawtooth base line. The probable cause is poor electrode technic or poor patient location. Repeat the line check, tighten the thumbscrews, and move the patient to another part of the room.

Wandering base line: Wandering base line is identified by rhythmic up-and-down motion of the base line. It is probably caused by motion of the patient or the electrodes. Check the patient for comfort and relaxation. See that the cable does not pull on the electrodes.

Irregular base line: Irregular base line is identified by a rough, jagged, irregular base line. The probable cause is poor electrical contact or metallic particles under the electrode. Wash the patient's skin, tighten the thumbscrews, clean the electrodes, and avoid perspiration.

Lubrication: The Sanborn Model 100 Viso is lubricated for life at the time of manufacture. Do not lubricate except as directed by an authorized Sanborn Company representative.

Balance control adjustment: After adjusting the stylus pressure, press the 1 MV button intermittently and then inspect the upper left-hand corner of each pulse. This corner may have a spike (underdamped), may be rounded (overdamped), or may be square (adjusted). If spiked, turn the damping control R 68 on the bracket behind the galvanometer magnet, lift the Lucite cover, and adjust for a square corner.

Trouble shooting: In most cases of trouble the fault is located in the operating technic, or the trouble may be caused by some easily replaced item such as a fuse. The trouble-shooting procedure below will save time in curing the difficulty:

1. Does the Viso work at all? See that it is plugged into a live power socket of the right voltage and frequency. Try replacing the power fuse behind the shield at the left-hand end of the cabinet.

2. Does the Permapaper refuse to feed properly? Most paper feed problems can be cured by taking out the paper and reloading. See that the power switch is turned to RUN.

3. When switching to a lead position, does the stylus go off-scale and drift on-scale 30 sec. later, with no EKG pulses, Viso apparently normal on STD position? Try replacing the patient fuse next to the patient cable socket.

4. Do one or more controls fail to work properly? Repeat the entire process of preparing the apparatus preliminary to use.

5. Is the recording of the electrocardiogram unsatisfactory? Some suggested cures are listed above.

6. Does it seem impossible to adjust for a dense, black, sharply defined base line? See the information on writing arm maintenance and repair.

7. If these procedures still do not cure the difficulty, then it is best to consult with the Sanborn Company, describing the problem, stating whether it is intermittent or continuous, giving the serial number of the Viso, and sending a sample of the defective recordings.

Chapter 111

Unipolar lead electrocardiography

JULIUS ELSON

In the last 20 yr. there has been a steadily increasing interest in and awareness of the importance of unipolar lead electrocardiography so that at present this method has been universally adopted. These leads have been shown to be advantageous for a number of reasons. Among the most important is the light that they have thrown on the significance of abnormalities of the Q waves, particularly in Lead III in the diagnosis of posterior wall infarction, the differentiation of pulmonary artery obstruction from posterior myocardial infarction, the diagnosis of bundle branch block, right ventricular hypertrophy, and the detection of abnormalities when the standard lead electrocardiogram is normal or shows questionable changes. The effect of changes in position of the heart are particularly demonstrable with these leads.

METHOD OF TAKING UNIPOLAR LEADS*

A unipolar lead is one that represents the heart voltages present at **one** particular spot on the body with respect to a nonfluctuating reference point. This reference point is secured by connecting the limb electrodes together through 3 equal resistances of 5000 ohms or more; the junction is the reference point or central terminal, which is usually embodied in the lead selector of the electrocardiograph or in a special switching box.

The electrodes placed on the body are then paired, in turn, with this reference point or central terminal to secure the unipolar leads which represent the actual voltages present at these single points on the body.

With limb leads, however, these voltages may be rather small, so that special steps must be taken to secure a record with complexes of sufficient height or amplitude for easy and accurate measurement.

Leads V_R, V_L, V_F: Leads V_R, V_L, and V_F are unipolar limb leads in which the pairing is achieved by placing an additional "exploring" electrode (normally attached to the C wire of the patient cable) on the limb being measured, slightly removed from the electrode already in place. Do not allow electrode jelly to communicate between electrodes.

With the lead selector switch placed on V, a unipolar limb lead will be secured. Special steps must be taken to produce a record with complexes of "readable" amplitude. Therefore, in taking these leads it is customary to increase the sensitivity of the electrocardiograph by 50%, or from 1 to 1.5 cm./mv. Allowance must be made for this increase since the amplitude of the waves will show a voltage of 1.5 times that which is actually present at the location being recorded.

"R" designates the right arm, "L" the left arm, and "F" the left leg (or foot).

Leads aV_R, aV_L, aV_F: Leads aV_R, aV_L, and aV_F are also unipolar limb leads and now are the ones most widely used. They are referred to as "augmented" unipolar limb leads.

No special placement of electrodes is required, nor is it necessary to change the sensitivity of the electrocardiograph to secure the increased or augmented height of the complexes. All necessary functions are performed automatically within the instrument or within a special lead selector box interposed between the patient and the electrocardiograph.

Turn the switch to the desired position—aV_R, aV_L, aV_F—to obtain the augmented unipolar limb leads.

The terminal letters designate the extremity and are paired with the central terminal or reference point.

Allowance must be made in measurement and interpretation for the 1.5 cm./mv. increased sensitivity.

Leads V_1, V_2, etc.: Leads V_1, V_2, etc. are unipolar chest leads. They are achieved by pairing a point on the chest wall (Fig. 710) with a neutral or zero reference point.

The subordinate numerals—V_1, V_2—are used to designate the position of the exploring electrode on the chest.

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*With permission of Sanborn Co., Waltham, Mass. From a bulletin entitled "Nomenclature and derivation of all presently-used leads" (1953).

Leads V_{3R}, V_{4R}, etc.: Leads V_{3R} and V_{4R} are unipolar chest leads, as described above, but are taken from positions on the chest wall to the right (R) of the sternum.

High V leads: High V leads are unipolar chest leads, taken with the chest or exploring electrode at positions on the chest wall one interspace above positions V_{1-6} and designated as high V_3, high V_4, etc.

V_E or V ensiform lead: V_E or V ensiform lead is a unipolar chest lead, taken with the exploring electrode placed on the chest wall at the ensiform (base of sternum) position.

Advantages of unipolar leads: Because of the method by which unipolar leads are obtained, their patterns can be explained in terms of easily understandable principles.

Since the indifferent electrode (of Wilson) is for practical purposes zero (though not absolutely so), the actual values of the potentials at each extremity can be determined with unipolar leads. This is in contrast to the conventional bipolar leads (the Leads I, II, III and the conventional chest leads), in which the potential recorded is the **difference** in the potential generated at each of the 2 electrodes in the bipolar leads.

In general, the relationships of the standard leads and the unipolar extremity leads are such that one type can be derived from the other as follows. The pattern of Lead I would be the potential values at the left arm (V_L) minus those at the right arm (V_R). The values of the potentials in Lead II would be the potentials recorded at the left leg (V_F) minus those at the right arm (V_R). The potentials recorded from Lead III would be the values derived from the left leg (V_F) minus those from the left arm (V_L). Thus it can be seen that the 3 standard bipolar leads consist of the algebraic summation of the unipolar leads.

Unipolar limb leads are useful to localize the altered electrical activity due to lesions of the various areas of heart muscle. Thus a unipolar lead from the left arm (V_L) records the altered potentials generated by a lesion in the left lateral wall of the heart; a unipolar lead from the left leg (V_F) records the effects of a lesion produced at the diaphragmatic (posterior) surface; the unipolar lead from the right arm reflects the potential variations of the heart muscle facing the atrial and ventricular cavities. Change in position of the heart with consequent altered relationships of the positions of these various regions of the heart with respect to the unipolar electrodes would alter the configurations of the electrocardiographic patterns in certain well-defined ways according to whether the heart is vertical, horizontal, or rotated either clockwise, counterclockwise, or backward or forward. It is beyond the scope of this section to describe and illustrate the different patterns of the unipolar leads according to the various positions of the heart and the modifications of these patterns produced by disease processes. This phase of electrocardiography has been thoroughly described in many books devoted to this subject.

Chapter 112

Interpretation of the electrocardiogram

JULIUS ELSON

THE NORMAL ELECTROCARDIOGRAM

The electrical activity in the heart muscle that precedes each contraction of the heart produces deviations in the electrocardiogram which are in general similar for all normal electrocardiograms. Nevertheless the electrocardiogram of every individual exhibits minor variations which are almost as characteristic for that individual as are his fingerprints. This is true only in so far as no alteration in the physiologic or anatomic state of the heart has occurred. The many variations of the normal electrocardiogram must be studied in order that proper interpretation of tracings may be made. Not infrequently erroneous conclusions with all their serious implications have been drawn by inexperienced persons who attributed minor deviations within the normal limits to organic heart diseases. Mistakes have also been made in the improper differentiation of abnormal tracings due to altered physiologic states from those due to actual organic disease.

Waves

The series of waves seen in electrocardiograms of normal heart cycles was arbitrarily named by Einthoven as the P, Q, R, S, T, and U waves. This is the conventional terminology that has been universally adopted.

The form, amplitude, and direction of the different waves vary in the different leads. The amplitude of the deflections in Lead II is equal to the sum of the amplitudes in Leads I and III. This knowledge is helpful in correcting errors that are sometimes made in labeling and mounting electrocardiograms of the conventional 3 leads.

The P wave is produced by electrical activity spread through the atria. It is a small, rounded deflection which varies in height from 0.5 to 3 mm., representing 0.05-0.3 mv. Duration or width of this deflection normally does not exceed 0.1 sec. At the end of this deflection, when the stylus has returned to the base line or isoelectric level, there is a period in which there is no deflection whatsoever from the isoelectric level until a group of sharper, taller waves, the QRS complex, appears.

The QRS deflections are due to the onset and spread of electrical activity throughout the ventricles. The interval between the onset of the P wave and the onset of the QRS complex is called the P-R or P-Q interval. The Q wave is often absent normally; under these circumstances the P-R interval is measured from the onset of the P wave to the onset of the R wave. If the Q wave is present, this interval is called the P-Q interval and is measured to the onset of the Q wave.

During this P-R interval the impulse is traveling through the atria to the ventricles along a set pathway known as the A-V node and the bundle of His. The P-R interval measures this time, which is normally from 0.12 to 0.2 sec. The QRS complex varies normally in certain respects—whether the initial deflection is directed upward or downward, whether the terminal deflection is downward (invert), or whether there are several upward or downward deflections. By convention the first deflection, if upright, is known as the R wave; if downward, the initial deflection is known as the Q wave.

The downward deflection that follows the R wave is known as the S wave. The S wave may often be absent. Not infrequently a second upward deflection occurs following the S wave. It is known as the R prime (R′) wave. If there is still another downward deflection following the R′ wave, it is called the S prime (S′) wave.

Since the QRS complex represents the spread of the action current throughout the ventricular musculature from start to finish, the time interval from the onset of QRS, whether it begins with a Q or with an R, to the end of its final deflection, either with an R or with an S, measures the speed of this electrical impulse through the ventricles. Normally this time interval does not exceed 0.1 sec.

The R wave normally varies in height from 5 to 15 mm. After the electrical disturbance represented by the QRS has subsided, there is a segment, known as the S-T segment, that is usually isoelectric and

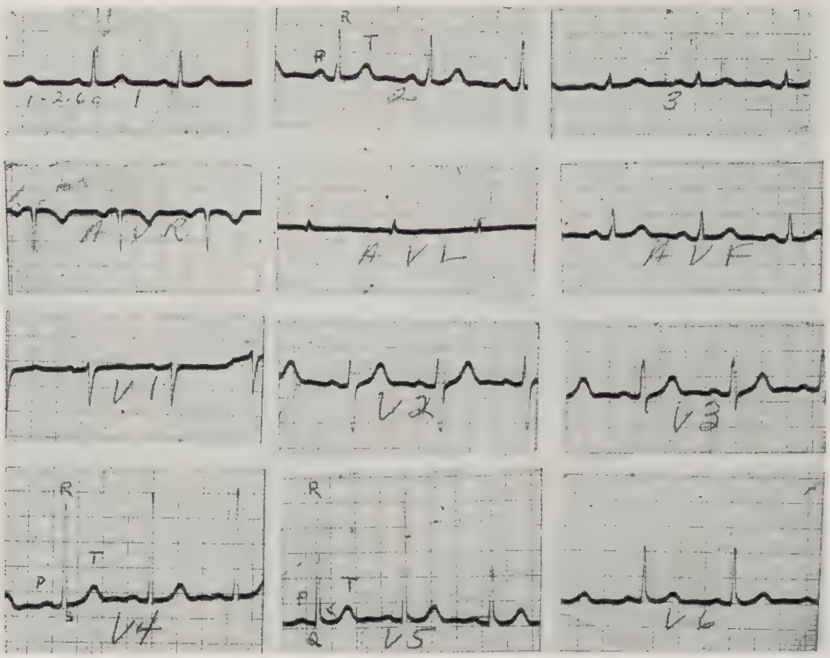


Fig. 711. Normal electrocardiogram. P, Q, R, S, and T waves appear in proper relationship and are of normal configuration and amplitude. Note that the amplitude of T in Lead II approximates the sum of the amplitudes of T in Leads I and III. Note also that Q and S may be absent normally.

of rather variable length; it ends in a rounded wider deflection known as the T wave. The ventricles are depolarized for the duration of the S-T segment and have not begun to recover their state of electrical excitement. This duration varies greatly, depending upon heart rate, age, and sex.

The interval from the beginning of the QRS complex to the end of the T wave is known as the Q-T interval. It represents the duration of the spread of the electrical excitation and then the recovery from this state of the ventricular musculature. This varies with heart rate, age, and sex, but for normal rates it is usually about 0.4 sec. The T wave, which is the final evidence of electrical imbalance in the ventricles, represents the phase of recovery from electrical excitation in the ventricles. During the period of the T wave the musculature approaches and reaches the completely polarized state. At the point of complete polarization the T wave has receded to the isoelectric level, and there are no deflections because the electrical potential is equal throughout the ventricles. The T wave in normal electrocardiograms is upright in Leads I and II, but in Lead III (called the "lead of exceptions" because of its variability under normal conditions) it may be upright or inverted. The amplitude of the T wave for the conventional leads varies from 1 to 6 mm.

A low upward wave often shortly follows the T wave. This is known as the U wave. Thus far the cause of this deflection has not been definitely ascertained. The U wave occasionally is inverted. When large or asymmetrical, it is sometimes associated with myocardial damage or certain electrolyte disturbances.

The chest or standard bipolar and most recently unipolar precordial leads have come into common clinical usage since 1932. They have been invaluable in the diagnosis of myocardial infarction. Two electrodes are used to take bipolar precordial leads. One, the exploring or chest electrode, is placed in various positions to be described. The other, the indifferent electrode, is placed over a point distant from the heart.

There are various types of bipolar chest leads, each designated according to the position of the chest (exploring) electrode and the indifferent electrode. These various positions of the electrodes are designated as follows: The Roman numeral IV is used to indicate a chest lead, and a letter follows the numeral IV to indicate the position of the indifferent electrode. If the indifferent electrode is on the left leg, the lead is called IV F; if on the right arm, it is IV R; if on the left arm, IV L; and if on the back, IV B. Another type of lead that is preferable is that advocated by Frank Wilson and his co-workers. It is a central terminal lead or V lead. In this lead the indifferent electrode is formed by connecting the right arm, left arm, and left leg electrodes together. This type of connection is advantageous in that the potential of this indifferent electrode is more nearly zero than when the indifferent electrodes are placed otherwise, where the current from the heart exerts some influence on the distant electrode though not nearly so much as on the exploring or chest electrode.

The chest lead may be recorded with the exploring electrode placed over different areas of the chest in relation to the heart. These positions on the chest are designated by numerals following the letters indicating the type of chest lead. They include the first letter C to indicate a chest lead, fol-

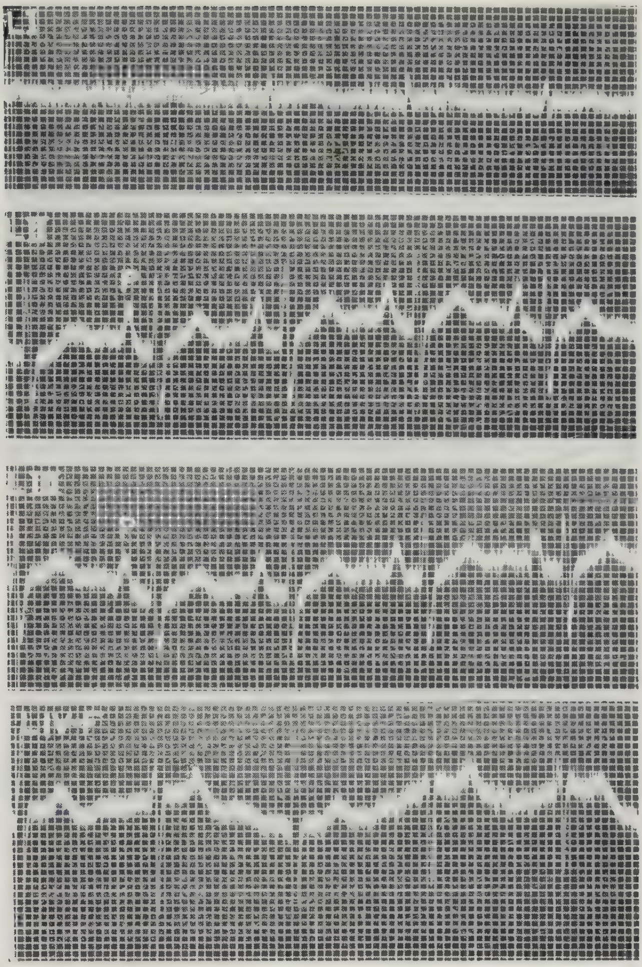


Fig. 712. Artifacts due to muscular tremors. In addition to the artifact characterized by fuzziness of the tracing due to muscular tremor, note the prominent sharply peaked P waves in Leads II and III characteristic of the so-called P-pulmonale. (Taken with a Cambridge galvanometer, using photographic equipment.)

lowed by the letters F, R, L, B, or V to indicate the position of the indifferent electrode as described above. In the case of the unipolar chest leads only the letter V is used as a prefix to the Roman numerals, signifying the position of the exploring electrode on the chest wall as described below.

If the chest electrode is placed in the fourth intercostal space just to the right of the sternum, the numeral 1 then follows. If the position is the fourth interspace just to the left of the sternum, the numeral 2 is used. When the position of the chest electrode is midway between that of numeral 2 and the midclavicular line in the fifth interspace, it is designated as 3. Position 4 is the fifth interspace, midclavicular line; position 5 is at the same horizontal level in the left anterior axillary line; position 6, the same horizontal level as 5, left midaxillary line.

If, for example, in the case of bipolar chest leads, the indifferent electrode is placed on the left leg, the various chest lead positions are recorded as CF_1, CF_2, CF_3, CF_4, CF_5, and CF_6, respectively.

To clarify these designations, let us emphasize the distinction between the leads CF_4 and IV F. With the first designation, the chest electrode is always in the fifth interspace, left midclavicular line; with the latter the chest electrode is placed over the heart apex wherever it may happen to be.

Various positions on the chest are utilized because it has been found that some lesions of the myocardium are more likely to be detected when the chest lead is placed in one position than when placed in another. Positions for the chest lead other than those previously described may sometimes be advisable when attempting to localize the site of the lesion or to determine the type of bundle branch block, if present.

As stated above, esophageal leads are sometimes desirable. In these instances a specially designed exploring electrode is swallowed until it reaches the level of the heart. The P waves are prominent in esophageal leads; for this reason this lead may be decisive in determining the nature of an arrhythmia in which P waves cannot be definitely identified in the conventional leads. This lead may also be of value in identifying posterior myocardial in-

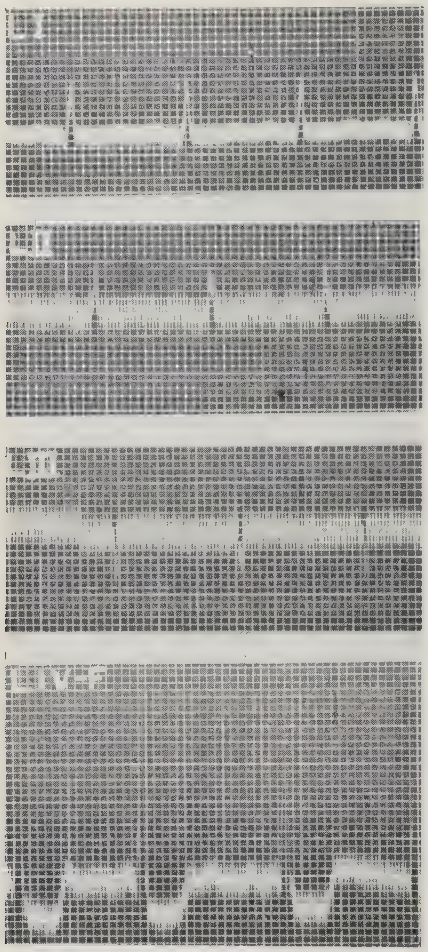


Fig. 713. Artifacts due to interfering alternating current. This is evident chiefly in Leads II, III, and IV F. (Taken with a Cambridge string galvanometer using photographic equipment.)

farction since the esophagus is more nearly in apposition to the posterior ventricular wall.

The appearance of the normal tracing taken with the chest leads varies in many details with the position of the electrodes. It is important, therefore, that the electrocardiographer familiarize himself with these normal variations. Since these variations are discussed in full in books devoted to electrocardiography, they are not presented in this chapter. The characteristics of Lead IV F are briefly described because Lead IV F is the one that has been used in taking many of the electrocardiograms reproduced in this chapter. In the last several years the unipolar chest leads (V leads) have generally replaced bipolar chest leads in common usage. What is said in describing the characteristics of the bipolar chest leads largely applies to the analogous unipolar chest leads (V_4).

Characteristics of waves in Leads IV F and V_4: The P wave is usually quite small and may be inverted, diphasic, or upright.

The P-R interval is as described for the conventional leads.

The QRS complex is usually of greater amplitude and tends to be slightly greater in duration than in the conventional leads. If R is less than 1 mm. in height, it is considered definitely pathologic. The S may be greater in amplitude than R, normally.

Q is usually absent, but when present, it is not greater than 2 mm. in depth. A Q wave deeper than this is usually significant of myocardial damage as a result of coronary artery involvement. If a Q wave is present in Lead I, there tends to be a Q in Lead V_4.

The S-T segment may be short or absent and normally is often elevated to a slight degree.

The T wave is characteristically larger than in the conventional leads, usually 5-7 mm., and is almost always upright in the adult. In young children the T wave is not infrequently inverted. Although it has been stated that the T wave is rarely notched or diphasic, it has been the author's experience on occasions to find notched and even slightly diphasic and inverted T waves in the absence of organic heart disease.

The U wave is often quite prominent, and at times its appearance shortly after the T wave gives a simulation of a terminal diphasic tendency to the T wave.

In V_1, T is normally often inverted, R is usually of low amplitude, and S is relatively deep. In V_4, which is taken with the electrode in the midclavicular line, fifth interspace, the appearance is the same as in IV F with the electrode over the apex, provided the apex is in its usual position in the midclavicular line, fifth interspace. Leads V_5 and V_6 give an appearance similar to that of Lead I except that the amplitudes of R, S, and T tend to be smaller. The rule is that with the exploring electrode to the right of the sternum the tendency is for R to be small, S to be deep, and T to be inverted; and as the electrode is moved to the left axillary line the R becomes relatively tall, the S relatively small, and the T upright.

Common technical difficulties

In order that a proper electrocardiogram be recorded it is necessary, when making the tracing, to understand some of the common technical difficulties that may cause artifacts or external deformities of the record, making proper interpretation difficult or impossible.

Deformities due to muscular movement of patient: The movement of an arm or leg may cause slipping of the electrode over the skin, with sudden marked or slight shifting of the stylus from the base line. Slighter muscular contractions such as muscular tremor produce smaller, very irregular vibrations in the stylus.

Deformities due to changes in skin resistance and polarization of electrodes: These are characterized by a more gradual undulating shifting of the base line.

Deformities due to improper grounding of electrocardiograph and interfering induction currents: These artifacts are characterized by regular vibrations due to 60-cycle alternating current at a rate of 60 times a second.

Deformities due to a loose string (applicable only

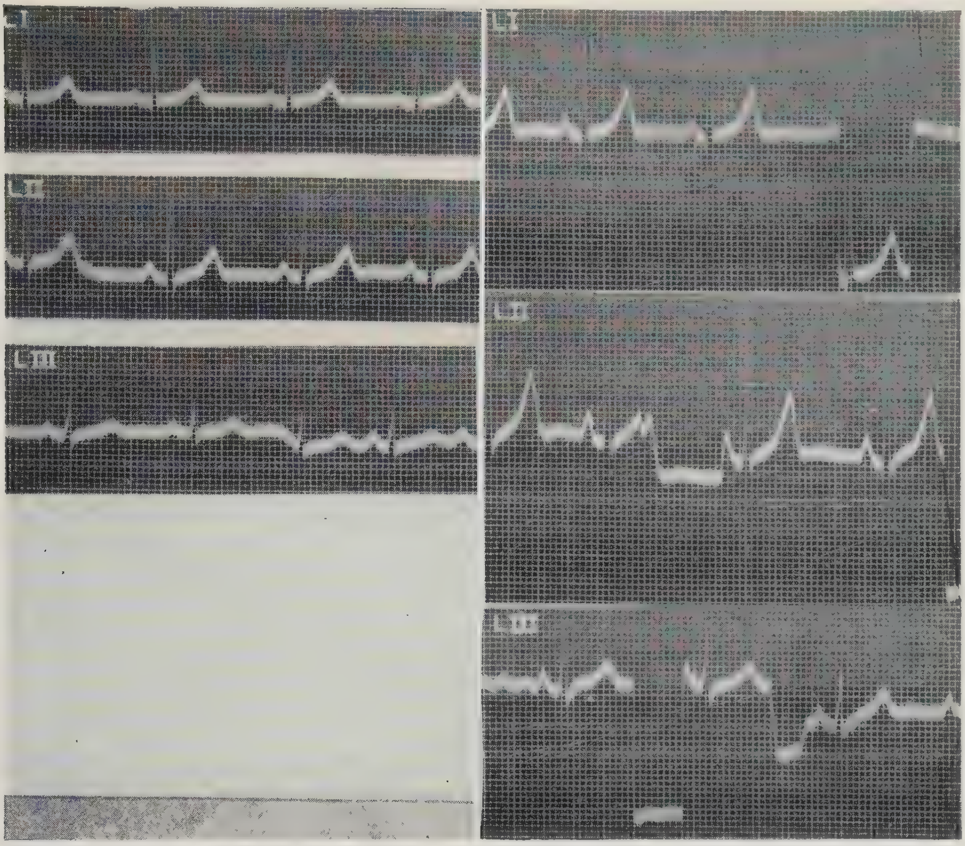


Fig. 714. Artifacts due to a loose string. In **A** the electrocardiogram has been properly standardized. In **B** the string has been overstandardized, the string being much too loose. Note that the P, QRS, and T waves are altered in size and form. (Taken with a Cambridge string type electrocardiograph using photographic equipment.)

to string type of electrocardiograph): If the resistance of the skin is too high, the string must be loosened to an unusual degree in order to standardize it. This prevents the string from stopping abruptly when a millivolt of current is added to the circuit so that the string "overshoots" or deviates above or below the proper amplitude. This would result in abnormally high amplitudes, slurring, and widening. When this occurs, the electrodes must be reapplied. The skin must be prepared more thoroughly, using electrode jelly to reduce skin resistance further. Overshooting must be watched for when taking each lead of the string galvanometer instrument.

Deformities due to loose contacts: Loose contacts at any place in the circuit may also cause sudden shifting of the base line.

Measurements of amplitude, heart rate, intervals, and duration of complexes

The duration of the P wave is measured from its convex surface at the instant it rises or falls from the base line to the moment it returns to this level. The duration of the QRS complex is measured from the beginning of the Q wave on its convex surface to the end of the R or S wave at its convex surface. The convex surface is used for making all other interval measurements such as P-R or P-Q, Q-T, and T.

The amplitudes of the upright deflections are measured from the upper surface of the base line to the upper surface of the topmost portion of that wave which is being measured. The inverted waves are measured from the lower surface of the isoelectric level to the apex at its lower surface. Duration and voltage of a complex are accepted as that measured in the lead in which they are the greatest.

Many ways of estimating the rates of the ventricles or atria have been described. A convenient method is to count the number of time lines lying in the space of 4 cycles, either atrial or ventricular. Then 6000 is divided by this figure; the result gives the rate of the atria or ventricles, according to which complexes were measured. Since the time lines are divided into groups of 5 by each heavy line (see above), it is convenient to begin measuring the 4 complexes where the first one falls on a heavy time line and then to count the heavy time lines, each representing a group of 5, until the fourth complex is reached, and figure accordingly.

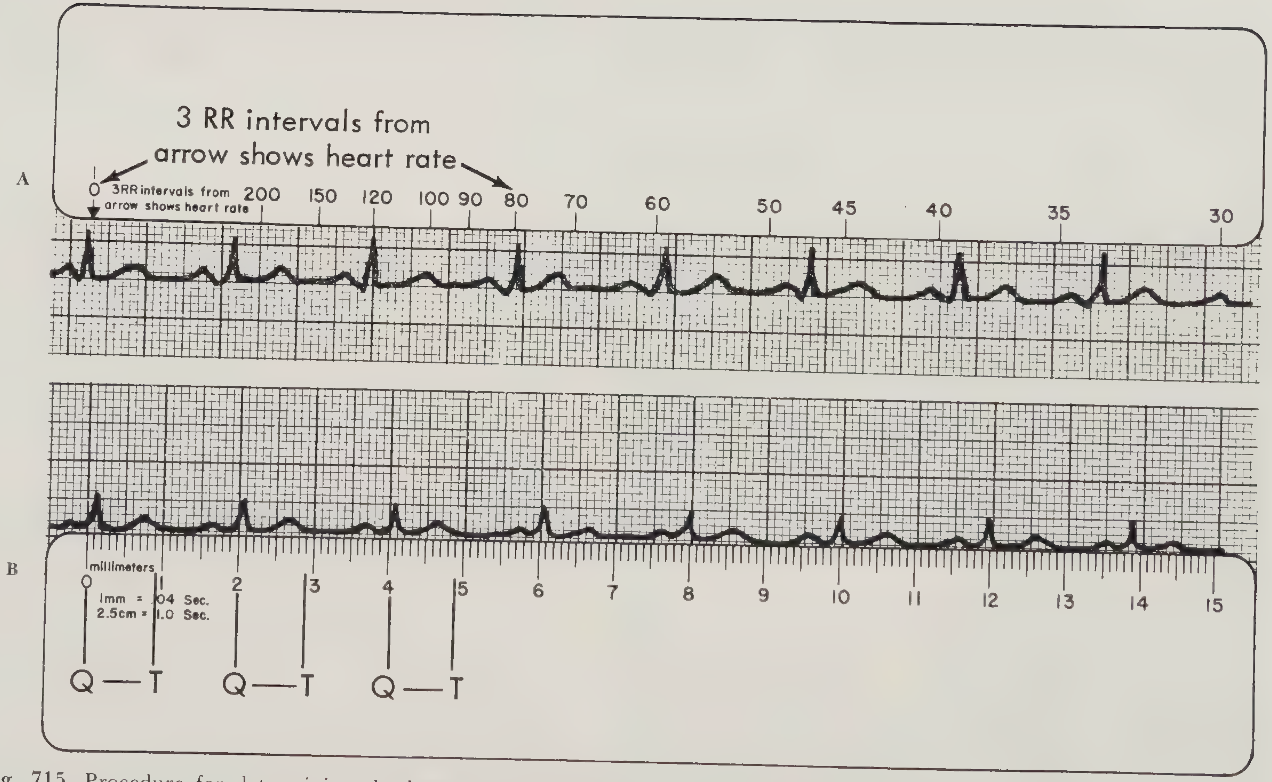
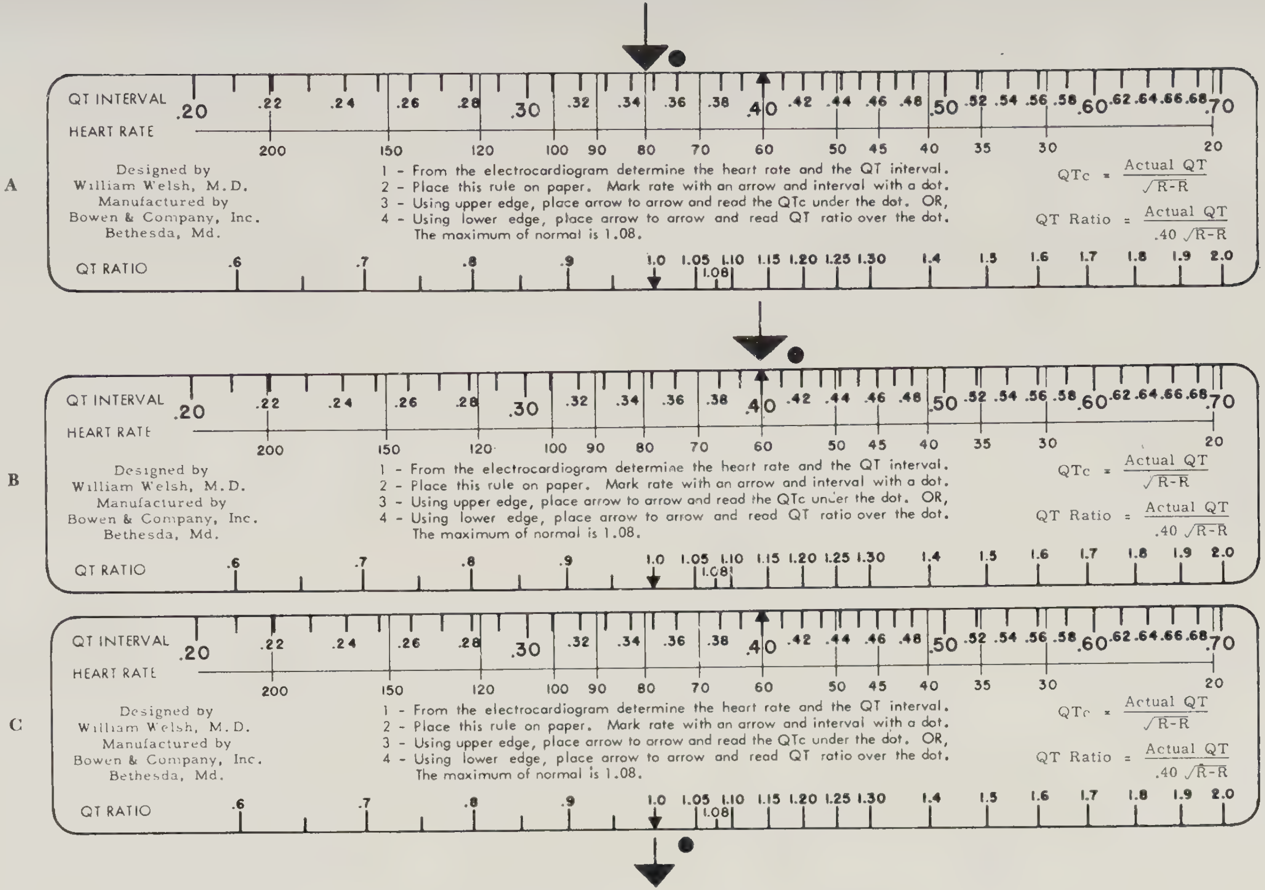

A
3 RR intervals from arrow shows heart rate
0
3RR intervals from arrow shows heart rate
200
150
120
100
90
80
70
60
50
45
40
35
30
B
millimeters
0
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
1mm = .04 Sec.
2.5cm = 1.0 Sec.
Q—T
Q—T
Q—T

Fig. 715. Procedure for determining the heart rate and Q-T interval from the electrocardiogram. **A,** Determine the heart rate by placing the arrow at the beginning of the rate measuring scale on the peak of an R wave and measuring off 3 R-R intervals. The ruler shows the heart rate to be 80. **B,** Determine the Q-T interval by using the millimeter scale and measuring the distance from Q to the end of T. In the example this is 9 mm. Because the paper speed is 1 mm./0.04 sec., it is necessary that the measured distance be multiplied by 0.04. The Q-T interval is therefore 9 × 0.04 or 0.36 sec. (Courtesy Bowen & Co., Inc., Bethesda, Md.)


A
B
C
QT INTERVAL
.20 .22 .24 .26 .28 .30 .32 .34 .36 .38 .40 .42 .44 .46 .48 .50 .52 .54 .56 .58 .60 .62 .64 .66 .68 .70
HEART RATE
200 150 120 100 90 80 70 60 50 45 40 35 30 20
Designed by
William Welsh, M.D.
Manufactured by
Bowen & Company, Inc.
Bethesda, Md.
QTc = Actual QT / √R-R
QT Ratio = Actual QT / .40 √R-R
QT RATIO
.6 .7 .8 .9 1.0 1.05 1.08 1.10 1.15 1.20 1.25 1.30 1.4 1.5 1.6 1.7 1.8 1.9 2.0

1 - From the electrocardiogram determine the heart rate and the QT interval.
2 - Place this rule on paper. Mark rate with an arrow and interval with a dot.
3 - Using upper edge, place arrow to arrow and read the QTc under the dot. OR,
4 - Using lower edge, place arrow to arrow and read QT ratio over the dot.
The maximum of normal is 1.08.

The maximum of normal is 1.08.

Fig. 716. Procedure for obtaining the QTc and the Q-T ratio. **A,** Place the ruler on any piece of paper. Note that on the upper edge of the ruler there are 2 overlapping scales, one marked "Q-T Interval" and the other "Heart Rate." Mark an arrow on the paper above the determined heart rate and a dot above the determined Q-T interval. **B,** The QTc is obtained by placing the arrow on the upper edge of the ruler under the arrow on the paper and reading the QTc under the dot. The QTc in the example is 0.416. **C,** The Q-T ratio is obtained by placing the arrow on the lower edge of the ruler above the arrow on the paper and reading the Q-T ratio over the dot. The Q-T ratio in the example is 1.04. A marked elevation shows heart disease, just as does a prolonged P-R interval or QRS interval. (Courtesy Bowen & Co., Inc., Bethesda, Md.)

Procedure for determining the heart rate and Q-T interval*

Another commonly used simple method employs a calculating ruler designed by William Welch, M.D.* This ruler has the added advantage of affording a simple method of measuring Q-T intervals and Q-T ratios.

Q-T calculator: The Q-T calculator is a calculating ruler to correct the Q-T interval for the heart rate.

Q-T ratio: The Q-T ratio is the ratio between what the Q-T interval is and what it should be. What it should be is expressed by Bazett's formula:

$$\text{Q-T} = 0.4\sqrt{\text{R-R}}$$

The Q-T ratio is therefore:

$$\frac{\text{Actual Q-T}}{0.4\sqrt{\text{R-R}}}$$

To calculate R-R from the heart rate, as determined from the electrocardiogram, and then to calculate the Q-T ratio is a simple slide rule procedure. The calculating ruler is, in effect, a preset slide rule to solve this formula.

Corrected Q-T (QTc): The corrected Q-T is expressed according to the following formula:

$$\frac{\text{Actual Q-T}}{\sqrt{\text{R-R}}}$$

It is readily seen that this is equal to Q-T ratio × 0.4. The ruler will also solve this formula.

An increase in the Q-T ratio or the QTc has diagnostic significance. Either should be determined as a matter of routine.

Procedure for determining heart rate and Q-T interval from electrocardiogram: Determine the heart rate by placing the arrow at the beginning of the rate-measuring scale on the peak of an R wave and measuring off 3 R-R intervals. See Fig. 715, *A*, in which the ruler shows the heart rate to be 80.

Determine the Q-T interval by using the millimeter scale and measuring the distance from Q to the end of T. In the example this is 9 mm. Because the paper speed is 1 mm./0.04 sec., it is necessary that the measured distance be multiplied by 0.04. The Q-T interval is therefore 9 × 0.04 or 0.36 sec. (Fig. 715, *B*.)

Procedure for obtaining QTc and Q-T ratio: Place the ruler on any piece of paper. Note that on the upper edge of the ruler there are 2 overlapping scales, one marked "QT interval" and the other "heart rate." Mark an arrow on the paper above the determined heart rate and a dot above the determined Q-T interval. (Fig. 716, *A*.)

The **QTc** is obtained by placing the arrow on the upper edge of the ruler under the arrow on the paper and reading the QTc under the dot. The QTc in the example is 0.416. (Fig. 716, *B*.)

The **Q-T ratio** is obtained by placing the arrow on the lower edge of the ruler above the arrow on the paper and reading the Q-T ratio over the dot. The Q-T ratio in the example is 1.04. (Fig. 716, *C*.)

The maximum of normal is 1.08.

A marked elevation is indicative of heart disease, just as is a prolonged P-R interval or QRS interval.

ELECTROCARDIOGRAPHY IN DISEASE AND IN DISORDERED PHYSIOLOGIC STATES

The electrocardiogram can be altered in many respects both by disease processes localized to the heart itself and by general constitutional disorders. Although many of these disease states cause electrocardiographic alterations that are nonspecific for that disease, there are others that are pathognomonic or highly suggestive of disease. The picture of acute myocardial infarction, for example, is usually characteristic. The location of this lesion can often be ascertained with a high degree of accuracy from the electrocardiogram alone. On the other hand, degenerative changes involving the myocardium, general constitutional disease, toxic agents, and even psychogenic factors may produce alterations in the electrocardiogram that are indistinguishable from each other.

The most significant changes in the electrocardiogram are those involving the QRS and T waves; in other words, those involving ventricular contraction. The QRS and T both comprise what is referred to as the "ventricular complex." The size, shape, direction, and duration of these complexes depend on several factors. The relative thicknesses of the right and left ventricular muscles, for instance, determine whether the direction of the main deflection of QRS is up or down in Leads I or III. Certain diseases of the cardiovascular system alter the relative thickness of the right and left ventricles in characteristic fashion. The origin of the impulse which the muscle, atrial or ventricular, receives also determines the size, shape, and duration of the complexes. If, for instance, the impulse stimulating the atria to contraction arises in the atrium in a point other than the normal location, the P wave will be altered in direction, size, or shape, and in its time relationship to QRS as well, since the time necessary for the impulse to reach the ventricles will be altered. It is normal for the impulse stimulating ventricular contraction to arise from a point above the ventricular musculature proper. As long as this obtains, the QRS and T complexes maintain a form, size, duration, and direction that is usual for this patient. If, however, a stimulus should arise in the ventricular muscle itself, the shape, size, duration, and direction are altered in a bizarre fashion, depending on the location of the exciting impulse in the ventricles. The T waves alone may be altered by multitudinous causes ranging from drinking cold water, psychogenic influences, or drug effects to serious organic disease involving the ventricular muscle. Any condition involving the pathway of conduction from the atria to the ventricles may alter the duration of the P-R or P-Q interval.

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*Manufactured by Bowen & Co., Inc., Bethesda, Md. Method by permission of the manufacturers.

In the same way, any disease or toxic process that impedes the conduction of the impulse as it spreads through the ventricles will cause increased duration of the QRS complex. The level and form of the S-T segments may be altered in certain characteristic respects by disease or toxic influences. Disordered rhythms of the heart, usually involving abnormal sites of impulse formation, and occurring as a result of a wide variety of organic and functional disturbances of the heart, give characteristic electrocardiographic patterns.

It is not our purpose here to give in detail the many patterns of electrocardiographic alterations that may occur. The electrocardiogram in the main types of cardiovascular diseases only will be discussed. An understanding of the pathologic physiology of disease is necessary in order to interpret abnormalities of the electrocardiogram.

Disturbances in depolarization and repolarization of heart muscle are caused by numerous factors both physiologic and pathologic; often these are manifested merely by lowering or inversion of the T wave. This change in T wave may not be specific for any type of lesion and may be caused by factors ranging from psychogenic influences to fibrosis resulting from long-standing and progressive coronary occlusion. In many cases it is possible to gain valuable clues to the nature of the disease by making serial electrocardiograms. With certain physiologic states, toxemias, and drugs, the T-wave changes are reversible; with organic disease, such as fibrosis of the myocardium, the changes usually are permanent. Often when serial electrocardiograms are taken, the electrocardiograms undergo characteristic pattern changes that are pathognomonic of acute myocardial infarction.

Clues to the types of disease may be obtained if one understands the effects of disease in causing strain on the various chambers of the heart. Strain on the left ventricle, on the right ventricle, and on the atria, with the consequent increase in muscle mass, produces characteristic evidence in the electrocardiogram. Some diseases or drugs are likely to exert their effects primarily on the conduction system, so that the presence of increased P-R conduction time will often give a clue to the disease or drug involved.

Axis deviation

Many cardiac diseases involve changes in relative size of the right and left ventricles, with consequent effect on the "electrical axis" of the heart. This electrical axis may also be altered by changes in the position assumed by the heart in the chest cavity. Thick-chested, sthenic individuals with diaphragms that tend to be high often have hearts that lie transversely in the thorax, whereas slender individuals with long chests often have hearts that assume a more nearly vertical position with consequently a different electrical axis with respect to the conventional leads. Since deep inspiration alters the position of the heart, it therefore affects the electrical axis. This applies to changes in position of the heart occasioned by extracardiac lesions such as pneumothorax, fluid in the chest, pulmonary collapse, fibrosis with pull on the mediastinal structures, etc.

It thus becomes important to determine alterations in the normal electrical axis of the heart. When the largest deflection of QRS is upright (R) in Lead I and invert (S) in Lead III, this indicates the presence of left axis deviation. See Fig. 717. When the largest deflection of QRS is upright (R) in Lead III and invert (S) in Lead I, right axis deviation is present. See Fig. 718. Left axis deviation is present in any condition that causes relative enlargement of the left ventricle over the right.

Right axis deviation occurs when there is relative enlargement of the right ventricle over the left. A transversely placed heart in which the apex lies more to the left produces the picture of left axis deviation. A vertically placed heart tends to produce a picture approaching right axis deviation. In young children, when the right ventricle is relatively thicker in relation to the left, the picture normally more nearly approaches right axis deviation, but, with age, as the left ventricle thickens, the picture of left axis deviation may finally be produced.

Deep inspiration will often cause a deeply inverted deflection (S) in Lead III to disappear and abolish the picture of left axis

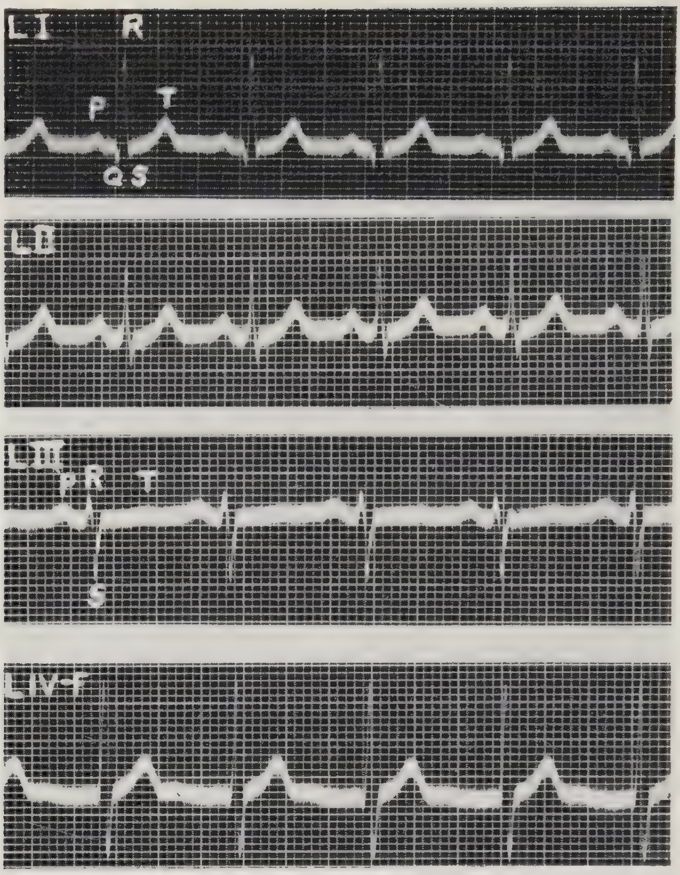

Fig. 717. Left axis deviation. Note that the chief deflection in Lead I (R) is upright and the chief deflection in Lead III (S) is inverted. A small R is present in Lead III. In more marked degrees of left axis deviation R in Lead III may be entirely absent. (Taken with a Cambridge string type of electrocardiograph using photographic equipment.)

deviation by producing a vertical position of the heart. If the right and left ventricles both enlarge and maintain their normal relationship with respect to size, then no change in electrical axis may occur. The occurrence of axis deviation can be helpful in determining the presence or absence and the type of valvular disease, since it is known that certain types of valvular disease tend to produce right axis deviation, whereas others tend to cause left axis deviation. The characteristic murmurs of these valvular deformities, or of congenital anomalies as well, may be absent or confusing; the finding of axis deviation suggestive of one or the other valvular condition may be decisive in the diagnosis. Changes in axis deviation associated with various types of cardiac pathology are mentioned in the discussion of electrocardiographic patterns found in the main classes of cardiac diseases. It is well to emphasize that left axis deviation may often be a normal occurrence. In these instances T_1 is upright, T_3 is inverted, and S_2 is absent or very small.

There are several methods for calculating the electrical axis of any electrocardiogram. The 3 most commonly used are (1) Einthoven's triangle, (2) the triaxial reference system, and (3) Dieuaide's chart. These methods are well described in monographs devoted to electrocardiography and its underlying theories.

Congenital heart disease

Although there is no absolutely specific electrocardiographic pattern for a congenital heart lesion with the exception of congenital dextrocardia, one can obtain from the electrocardiogram much information that together with other clinical data will establish the diagnosis.

The reason for this is that the electrocardiographic pattern reflects only what is going on in the heart muscle and is not produced by

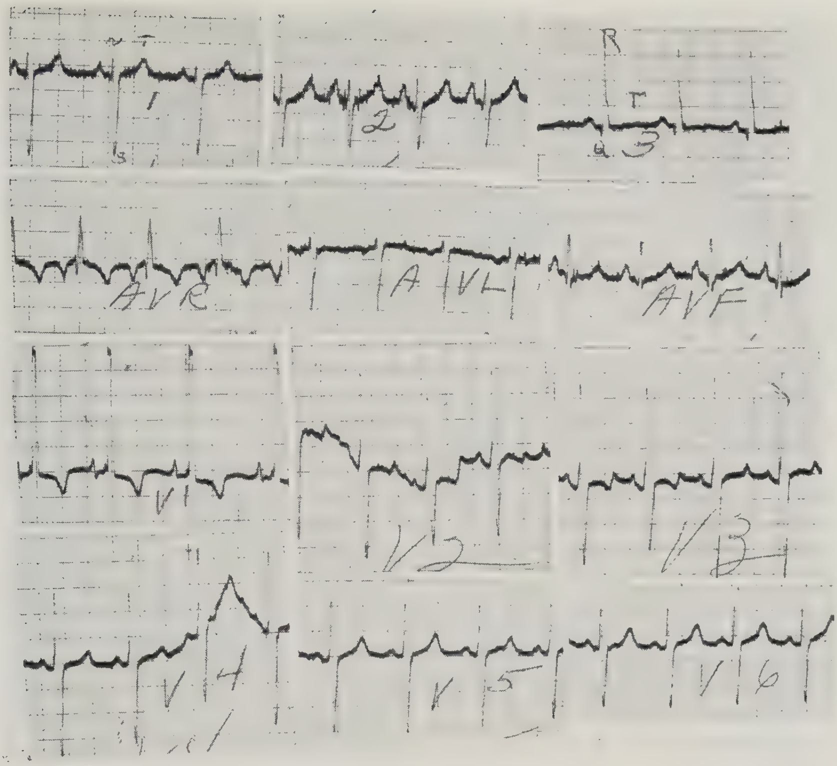

Fig. 718. Right ventricular hypertrophy and right axis deviation. Note the marked right axis deviation—low amplitude R (r) and deep S with upright T in Lead I; the small q and tall R in Lead III; the small q, prominent R, and small s in Lead aV_F; and especially the very tall R, depressed ST and inverted T in Lead V_1. Also note that R becomes progressively smaller and T less inverted and finally upright as the leads are taken over the left chest positions—V_2, V_3, V_4, V_5, and V_6. Taken from a patient with congenital cyanotic heart disease (tetralogy of Fallot).

the anatomic defect itself. Therefore, the type of abnormal electrocardiogram will depend on the character of the alterations in the hemodynamics with the resultant strain and enlargement of the various segments of heart muscle surrounding the individual chambers of the heart and a consequent change in position of the heart in relation to the electrocardiographic leads. The importance of the electrocardiogram in congenital heart disease has been particularly enhanced by the spectacular advances in surgical correction of certain defects. It is therefore of vital importance to be certain of the type of anatomic defect. This can be illustrated by citing its importance in helping to distinguish the ostium primum type of atrial septal defect from the ostium secundum type. In the latter there is usually right axis deviation and incomplete bundle branch block, whereas in the former there is usually a characteristic combination of left axis deviation in the extremity leads, and incomplete right bundle branch block together with right ventricular hypertrophy as shown in the chest leads. The prognosis is much more serious, and the surgical means of correcting the defect is more difficult in the ostium primum type.

The most striking and characteristic electrocardiographic changes occurring in congenital heart disease are those found in true congenital dextrocardia (right-sided heart). In this condition all the complexes in Lead I are inverted, Lead II resembles a normal Lead III, and Lead III resembles a normal Lead II.

Diagnosis of congenital dextrocardia may be made with absolute certainty from the electrocardiogram alone if the electrocardiographer can be sure that the technician who took the tracing did not reverse the arm leads, erroneously connecting the right arm lead wire to the left arm electrode and the left arm lead wire to the right arm electrode. Unfortunately this careless technical error is by no means a rare occurrence. It behooves all those who are responsible for making electrocardiograms to check carefully all lead wires several times to make sure that no such errors will be made.

Other congenital anomalies that commonly result in electrocardiographic changes are congenital pulmonary stenosis and defective interventricular septum. These conditions cause well-marked right axis deviation. The QRS complexes are of much greater amplitude than usual. Congenital heart block may occur in which the atrial complexes (P) have no constant relationship to the QRS complexes; in other words, the ventricles contract independently of the atria because the stimulus from the atria is blocked somewhere in the conduction pathway from the atria to the ventricles.

Rheumatic heart disease

Rheumatic fever may cause changes in the endocardium, chiefly the valves, the myocardium, or the pericardium, or changes may occur in all these structures at the same time. One of the most characteristic electrocardiographic effects of rheumatic fever in its acute stage is involvement by the acute inflammatory process of the atrioventricular conduction system. This results in delay in atrioventricular conduction with a subsequent increase in the P-R interval. This depression in conduction with consequent increase in P-R interval may be very slight, or it may become so marked that the ventricles do not receive any impulse at all from the atria. Thus they do not contract in response to atrial stimulation so that one may see P waves (atrial) without any regular relationship to their corresponding QRS complexes.

These varying degrees of increased P-R interval represent what is known as **heart block.** Heart block may be so marked as to be complete. If the block is complete, no ventricular complexes occur in response to the atrial wave. In complete heart block each of the ventricular complexes occurs independently of the atrial waves at a much slower rate than the latter. Milder degrees of heart block, in which each ventricular contraction responds to an atrial contraction, cannot be detected by ordinary clinical means. The electrocardiograph is invaluable in these conditions. It gives definite, objective, confirmatory evidence of the presence of acute rheumatic fever. Usually such P-R interval changes in acute rheumatic fever are transitory and disappear when the acute inflammation subsides. This must be distinguished from the P-R interval prolongation caused by digitalis, in which there are usually other tell-tale effects indicating digitalis action.

The inflammatory changes of rheumatic fever which involve the myocardium proper cause alterations in the electrocardiogram not

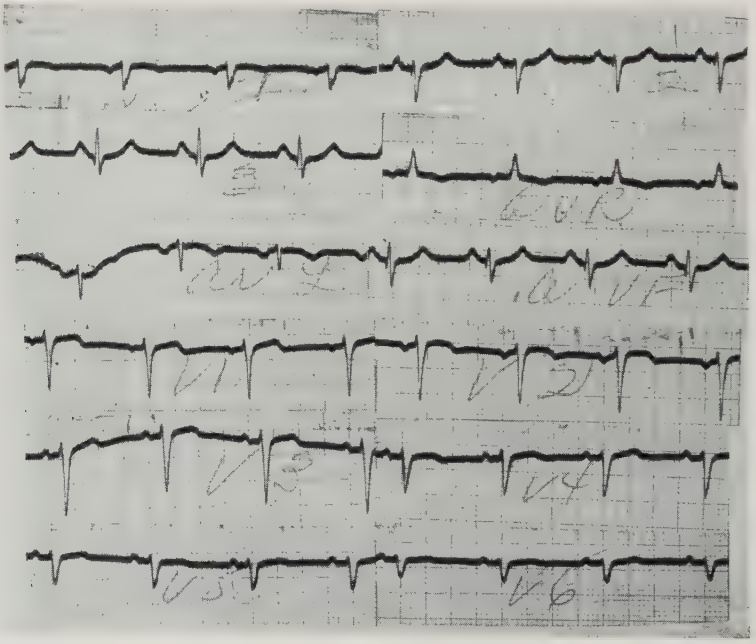

Fig. 719. Congenital dextrocardia. Note the pathognomonic inversion of all the complexes in Lead I. In the normal electrocardiogram Lead II would resemble Lead III, and Lead III would resemble Lead II.

so specifically associated with rheumatic fever as the P-R interval changes. Other types of pathologic processes may also cause these patterns. Such manifestations may be characterized by slurring or notching, lowering or widening of the QRS complex, lowering or inversion of the T waves, and changes in the Q-T intervals.

If the pericardium is involved by the rheumatic inflammatory process, there will likely be elevation of the S-T segments in Leads I and II and the chest leads. These changes are not specific for rheumatic fever since any process that alters depolarization by involving the pericardium in this way can cause such electrocardiographic patterns.

The electrocardiographic pattern that develops with chronic endocarditis and consequent valvular involvement depends upon the valve involved. Since the mitral valve is frequently stenosed, increased strain is placed on the chambers behind this valve, i.e., the left atrium, the right ventricle, and eventually the right atrium. The pattern of right axis deviation develops with increased right ventricular strain and increased muscle mass. Because the left atrial muscle so often becomes hypertrophied in mitral stenosis, the P wave is often altered, becoming prominent and notched. The atrial muscle is increased and a longer time is needed for the action current to traverse it; thus there is widening of the P wave. This type of P wave, characteristically present in Leads I and II, is quite suggestive of mitral stenosis.

A disorder of rhythm, known as atrial fibrillation, is very common in rheumatic heart disease with mitral stenosis because of the strain upon the left atrium. This is reflected in the electrocardiogram in a characteristic manner.

If the mitral valve involvement is such that regurgitation is prominent and the stenosis is relatively minor or absent, strain is put upon the left ventricle, and left axis deviation occurs. This is not so suggestive of rheumatic heart disease since many more pathologic heart conditions other than rheumatic heart disease may give left axis rather than right axis deviation. If the aortic valve is involved and causes regurgitation or stenosis of that valve, a frequent occurrence in rheumatic heart disease, strain is put primarily on the left ventricle. This ventricle hypertrophies and left axis deviation develops. If both mitral stenosis and aortic stenosis or aortic regurgitation are present, and if both right and left ventricles hypertrophy, then the electrical effects of right and left ventricular hypertrophy may counterbalance each other, and no axis deviation may result.

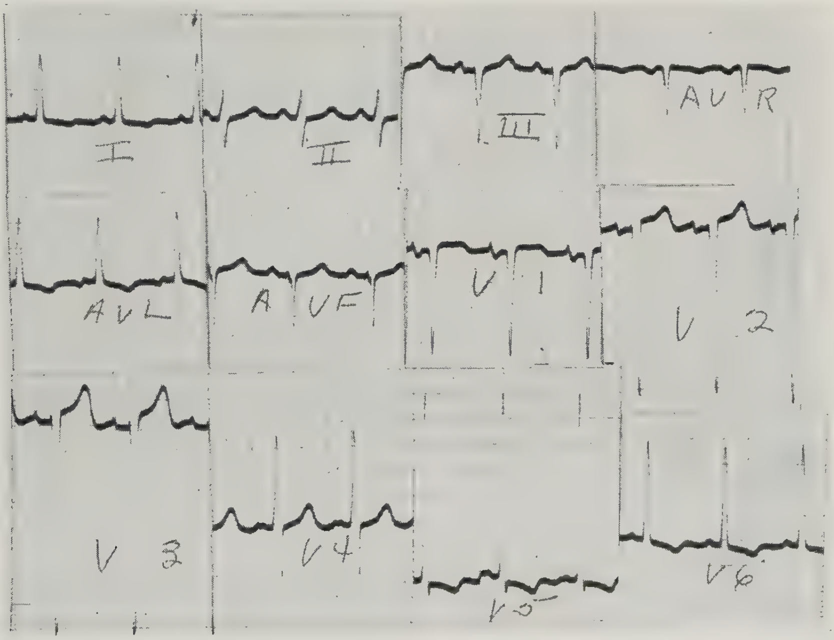

Fig. 720. Left ventricular strain and left ventricular enlargement. Note the prominent R and inverted T in Leads I and aV_L, the deep S and upright T in Leads III and aV_F, the very tall R, depressed ST, and inverted T in Lead V_5 and to a less extent in Lead V_6.

Hypertension

In hypertension, especially of a marked degree and of long standing, a rather typical electrocardiographic pattern of left ventricular strain develops. The R wave in Lead I is usually tall and the S-T segment becomes depressed and coved in Lead I and elevated in Lead III. The S wave is deep in Lead III, and the R wave is relatively small or absent in Lead III. The T waves in Leads I and III are in opposite direction from those of the main deflection of QRS in Leads I and III, i.e., inverted in Lead I and upright in Lead III. The QRS may be slightly widened in some instances due to the additional time required for the action current to traverse the thickened ventricular muscle. Fig. 720 is an example of left ventricular strain and enlargement.

Electrocardiography in coronary artery disease

General comments

The electrocardiogram does not record abnormalities in coronary arteries but only the electrical deviations caused by changes in the myocardium. In some instances, all too frequent, disease of the myocardium may be present but in such a location with respect to the electrocardiographic leads that the electrocardiogram remains normal.

Many electrocardiographic abnormalities seen in coronary artery disease are not specific and occur in other diseases. These may be alterations in rhythm, conduction, or the presence of left ventricular hypertrophy. Although T-wave inversion occurs quite uniformly as a result of myocardial ischemia, it is found not infrequently when ischemia is absent. Emotional factors, the drinking of cold water, indulgence in tobacco, hyperventilation, position of the patient, whether upright or recumbent, certain drugs, such as digitalis, quinidine, and morphine, and electrolyte disturbances all may alter the T wave. Thus, particularly when T-wave changes alone are observed, it becomes important to take into consideration all the above possible factors in trying to arrive at a conclusion concerning their clinical significance.

The diagnosis of myocardial infarction can be made on the basis of the electrocardiogram alone in the absence of other data only under certain circumstances. These are such that the QRS, S-T segment, and T-wave patterns go through certain characteristic evolutionary changes when serial electrocardiograms are taken. Indeed, the general extent and site of such infarction can be determined when such characteristic patterns are elicited. These characteristic serial evolutionary patterns are illustrated in Figs. 721-726.

Myocardial infarction

The electrocardiograph has been of great value in ascertaining definitely the presence of myocardial infarction. This condition is commonly referred to as **coronary thrombosis.** Since, however, the electrocardiographic patterns are caused directly by resulting actual necrosis of the heart muscle itself, that is, the infarction of the muscle, and not by the thrombus which obstructs the coronary artery, it is more correct to refer to these electrocardiographic changes as being due to myocardial infarction. This fact must be emphasized that, whereas most cases of myocardial infarction are due to coronary thrombosis, **occasionally myocardial infarction occurs in cases with extensive narrowing of the coronary arteries without thrombosis.**

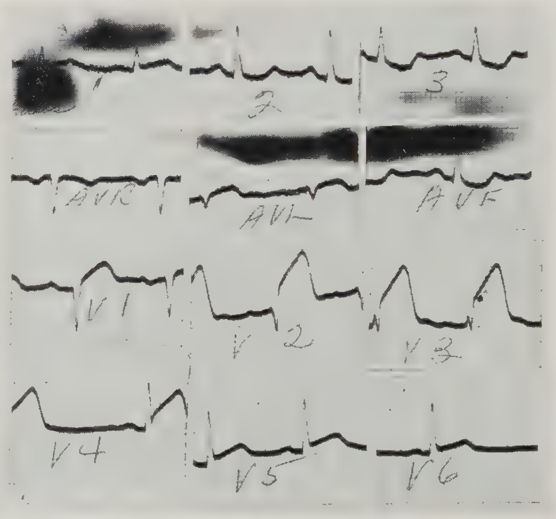

Fig. 721. Serial changes in acute anterior myocardial infarction. Electrocardiogram taken the day of the attack. The S-T segment is slightly elevated and has a suggestion of upward convexity in Lead I and depressed and sagging in Leads II and III. In Lead aV_L the R wave is almost absent, the S-T segment is upward bowed, and the T wave is somewhat elevated with slight terminal inversion. In Lead aV_F the S-T segment is reciprocally depressed and sagging as in Lead III. The most striking and diagnostic changes are in Leads V_2, V_3, and V_4 and to a small extent in V_5 and V_6. The R wave in Lead V_2 is almost nonexistent and very small in V_3; the S-T segment is almost absent with abrupt and markedly elevated take-off of the T wave in Leads V_2, V_3, and V_4 and to a relatively small extent in Leads V_5 and V_6.

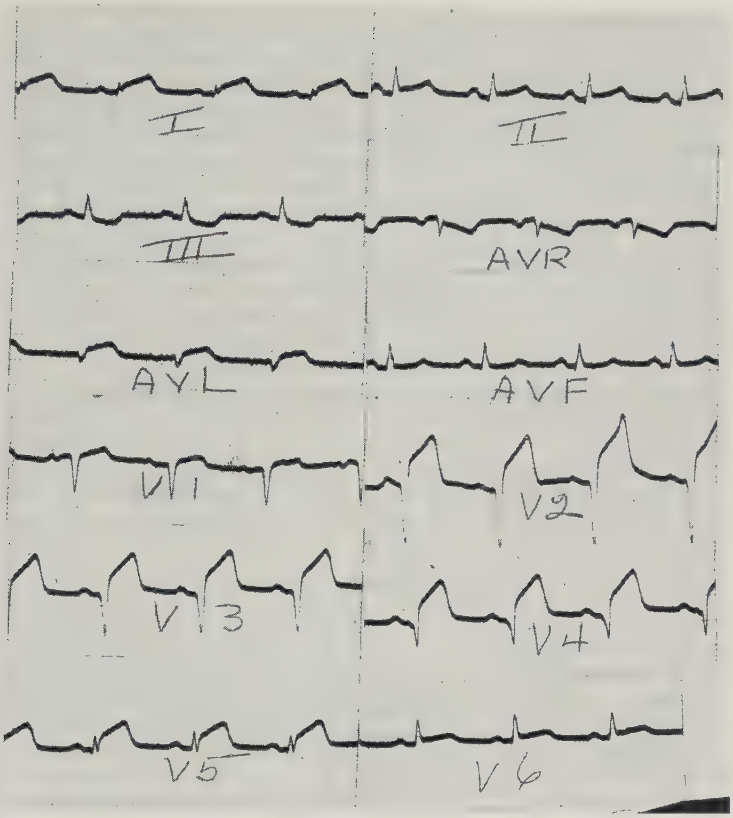

Fig. 722. Electrocardiogram of same patient as shown in Fig. 721 taken 7 days later.

The electrocardiographic picture of myocardial infarction varies according to the site and extent of the coronary artery obstruction. In general, there are 2 main varieties of coronary obstruction, anterior and posterior. These pictures may be modified by such factors as presence of previous infarctions, involvement of the conduction system, or involvement of the pericardium or endocardium. Posterior infarction is often referred to as "diaphragmatic" infarction since in these cases the area of ventricle involved faces the diaphragm.

Anterior myocardial infarction: Most commonly the left anterior coronary artery is obstructed, with resultant infarction of the anterior apical portion of the left ventricle. If serial electrocardiograms are taken, a characteristic series of changing electrocardiographic patterns will usually be noted. These changes are often referred to as "evolutionary" changes. The QRS complexes in Lead I are frequently small, slurred, and W shaped. The earliest (within the first 2 days) and most characteristic change is an elevation and upward convexity of the S-T segment in Lead I and aV_L, and a reciprocal depression of the same segment in Lead III. The T wave early has a high take-off from the S-T segment and may have merely a plateau type of configuration with perhaps a slight terminal dip. Later the S-T segment in Lead I drops steadily until it assumes a position at or slightly below the isoelectric level, maintaining a characteristically upward convexity.

The T wave in Lead I becomes completely inverted with a characteristic "coved" take-off from the S-T segment. The opposite changes occur in Lead III. The Q wave in Lead I, which has made its appearance early, will usually persist. The S-T segments and T waves occasionally may eventually resume a completely normal pattern. The characteristic residual patterns of an anterior myocardial infarction are the prominent Q and inverted and coved T waves in Lead I, with opposite configuration (normal) in Lead III. This is known as the $Q_I T_I$ pattern of anterior myocardial infarction.

Changes in the chest leads in anterior infarction are quite characteristic and frequently even more striking than in the other leads.

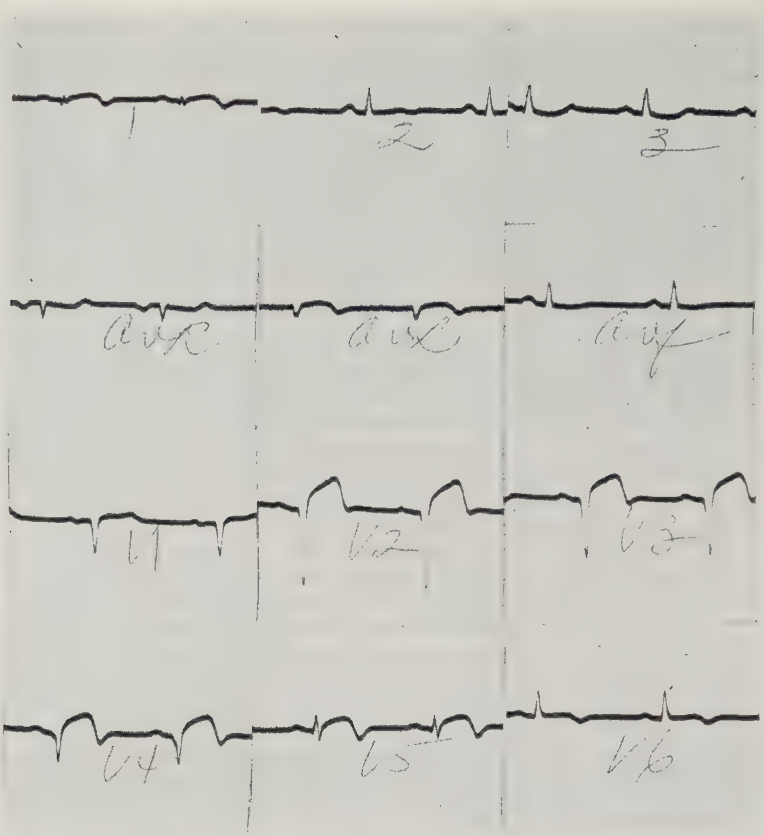

Fig. 723. Electrocardiogram of same patient as shown in Figs. 721 and 722 taken 12 days after the attack. A small Q is now present, R has become minute, the S-T segment has become almost isoelectric, and T has become inverted in Lead I; T has become inverted in Lead II and S-T sagging has become much less in Lead III. In Lead aV_L the changes resemble those in Lead I. The S-T deviations and high take-off of T have become much less in V_2, V_3, V_4, and V_5: T shows terminal inversion in V_2, V_3, and V_4 and is completely inverted in V_5 and V_6.

Occasionally, in this type of infarction, chest leads may be the only leads that reveal the changes characteristic of anterior infarction, thus emphasizing the value of these leads in this condition. In general, the changes in the left chest leads parallel those in Lead I except that they are often more marked. In the early stages the S-T segment is displaced upward and the initial deflection of QRS may be only very slightly upright or deeply inverted (deep Q). A deep Q in Lead V_4 is never normal and usually signifies myocardial infarction. The T in the early stages has an elevated take-off from the S-T segment that is short and abrupt and often exhibits a slight terminal dip. Gradually, the S-T segment becomes depressed until it reaches the isoelectric level or a point slightly below it.

The T wave becomes sharply and deeply inverted. The Q wave is characteristically prominent and is more definitely indicative of the presence of anterior infarction than an inverted T wave. Nevertheless a deep Q wave might never appear in Leads V_4, V_5, and V_6, and an inverted T wave in these left chest leads may be the only evidence of an anterior myocardial infarction.

If the initial deflection in Lead V_4 is upright but is only 2 mm. or less in amplitude, it is quite suggestive of the presence of myocardial infarction. If an inverted T wave as well is found, then the evidence of myocardial infarction is more nearly conclusive. Figs. 721-723 show the sequential changes, over a period of several weeks, in a case of acute anterior myocardial infarction.

Posterior or diaphragmatic myocardial infarction: Posterior or diaphragmatic myocardial infarction produces changes similar to those of anterior infarction in Leads I and III except that the patterns are reversed. The characteristic rise in the RS-T level takes place in Lead III and usually also in Leads II and aV_F, whereas reciprocal depression occurs in Lead I and often in the left chest leads. A prominent Q wave in Leads III and

aV_F, and often in Lead II becomes evident just as in anterior infarction a Q_I makes its appearance. There is the same upward rounding or convexity of the S-T segment, together with the elevation of this segment, the same stages of progressive lowering of S-T segment and inversion of T wave in Lead III resulting from acute posterior infarction as are found in Lead I in cases of anterior infarction. Variations from these classical patterns are seen. These depend upon the presence of the infarction in locations other than those described above, or they may be due to the fact that an old lesion has preceded the fresh lesion.

In the chest leads changes with posterior infarction are not striking. Occasionally a prominent R is present in V_I. The S-T segment may be depressed to varying degrees and may gradually rise to the isoelectric level. The T wave is normal or is occasionally unusually tall. Figs. 724-726 show the serial changes occurring in a case of acute posterior myocardial infarction.

More information relative to posterior infarction may be obtained by using esophageal leads since these leads are placed as near the posterior surface of the heart as it is possible to place a lead in a living subject.

Subendocardial myocardial infarction

In these cases the infarction is confined to the subendocardial region. Because there is no transmural (through and through) involvement, there is no Q wave, or at least the Q wave if present is not significantly abnormal. Characteristically, there are well-marked S-T depressions in the standard and chest leads. The RS-T segments may be elevated in Lead aV_R. Refer to Fig. 727.

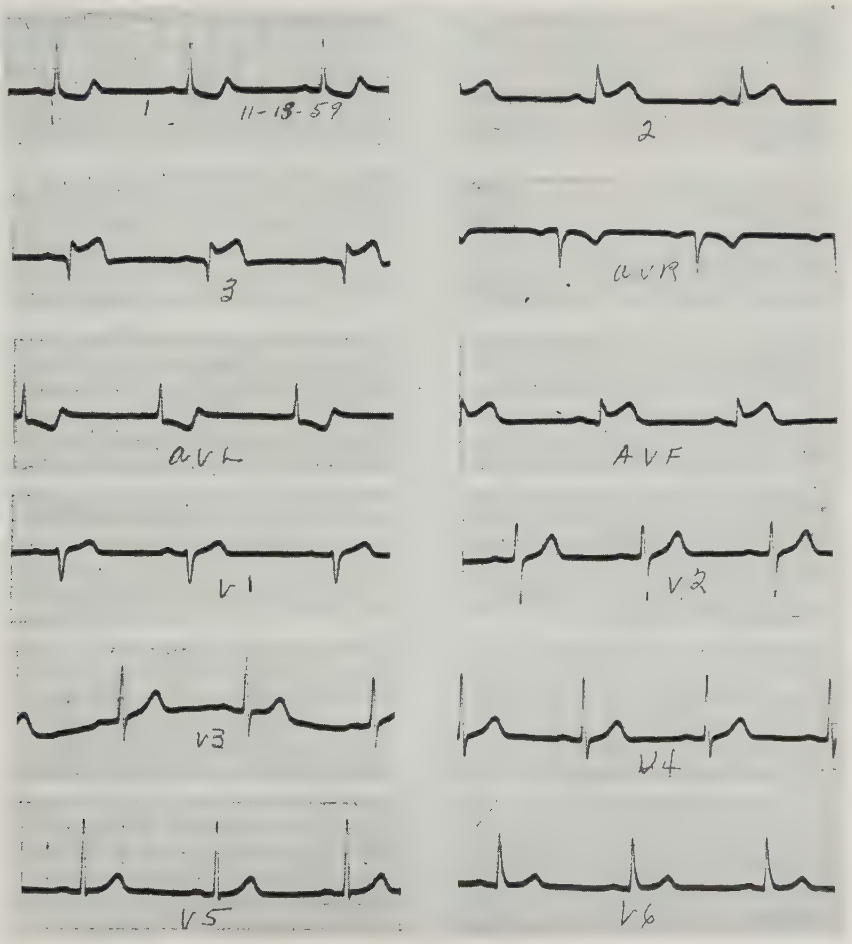

Fig. 724. Acute diaphragmatic (posterior) myocardial infarction. The characteristic changes occur in Leads II, III, and aV_F with reciprocal S-T deviations in Leads I and aV_L. This electrocardiogram was taken the day of the attack. Note that the S-T elevations are in Leads II, III, and aV_F. The Q wave is deep and the R wave small in Lead III.

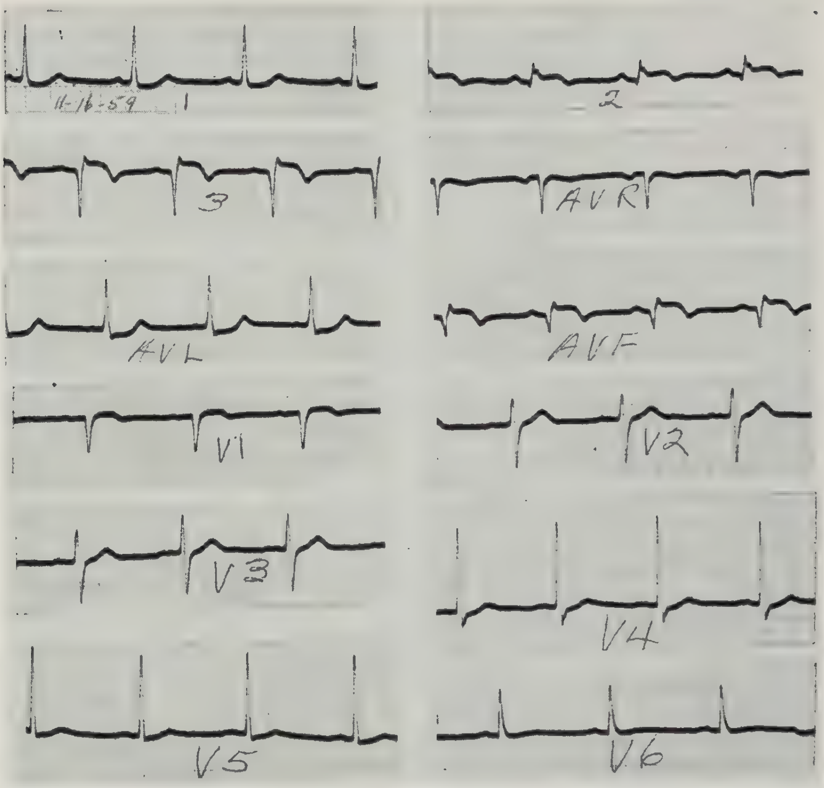

Fig. 725. Electrocardiogram of the same patient as shown in Fig. 724, 3 days after the attack. The Q wave in Lead II is somewhat more evident. T waves in Leads II and III are now inverted with less elevation of S-T. Changes in Lead aV_F resemble those in Lead III. The reciprocal sagging of S-T in Leads I and aV_L has almost completely disappeared.

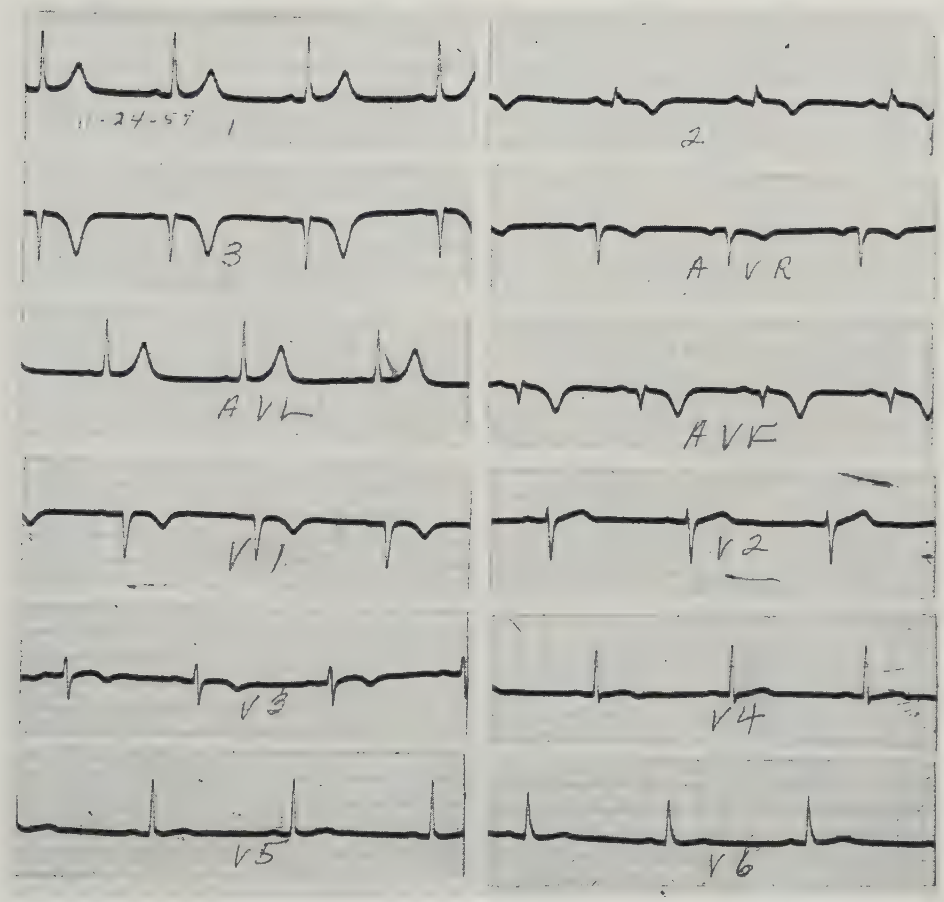
11-24-57
1
2
3
A V R
A V L
A V F
V 1
V 2
V 3
V 4
V 5
V 6

Fig. 726. Electrocardiogram of same patient as shown in Figs. 724 and 725 taken 11 days after the attack. The S-T segment has become almost isoelectric in Lead II and isoelectric in Leads III and aV_F. The T wave has become more deeply inverted in Leads II, III, and aV_F.

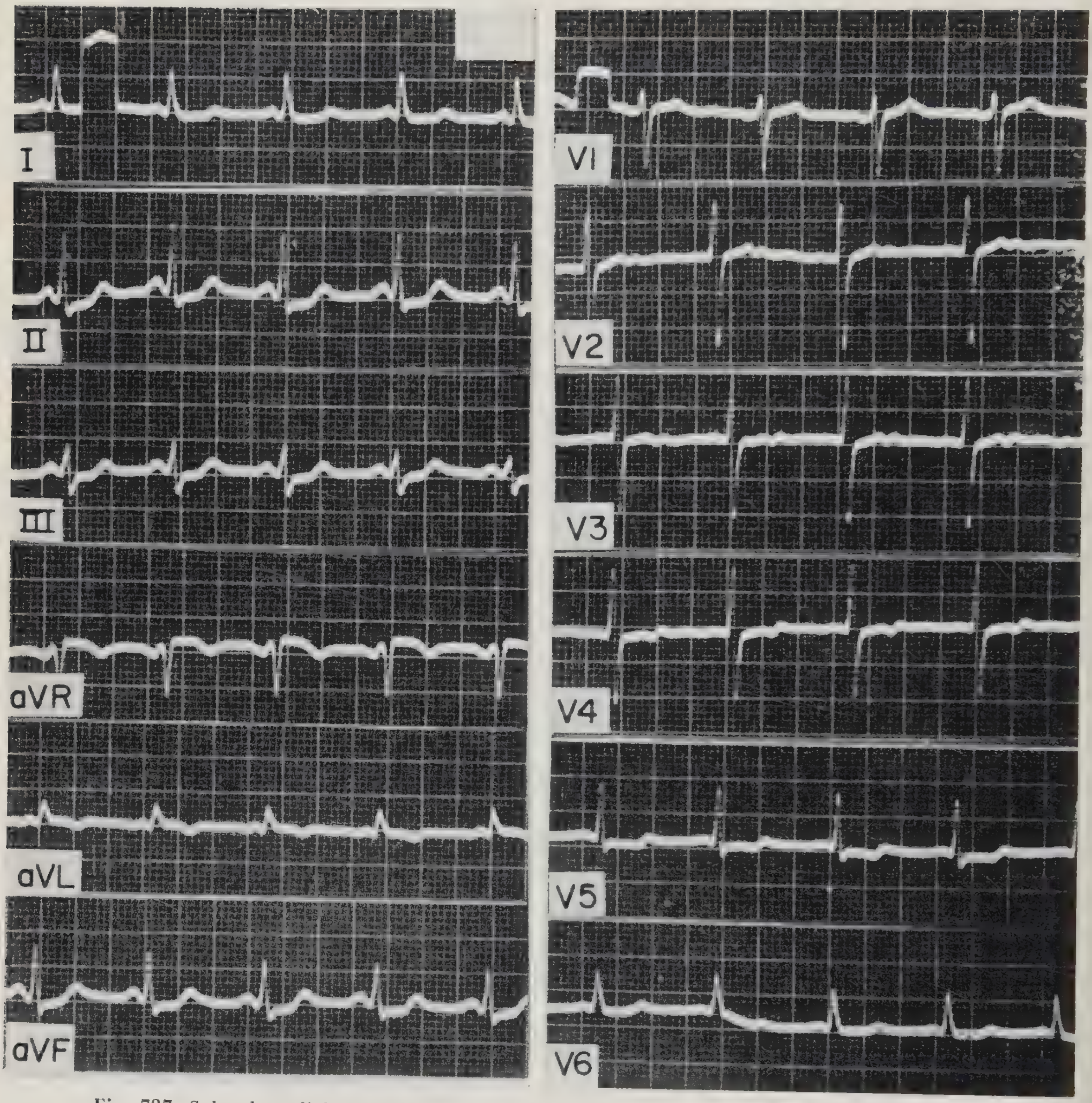
I
II
III
aVR
aVL
aVF
V1
V2
V3
V4
V5
V6

Fig. 727. Subendocardial myocardial infarction. (From Dimond, E. G.: Electrocardiography. St. Louis, 1954, The C. V. Mosby Co.)

Ventricular aneurysm

As has been described, the elevated S-T segment with high take-off of T and the subsequent evolutionary lowering of the S-T segment to the isoelectric level, together with subsequent complete inversion of T and the presence of a deep- or wide-notched Q, are highly characteristic of the serial patterns of acute myocardial infarction. Occasionally, the S-T segment elevation and high take-off of T will persist as a permanent pattern. When this happens, the diagnosis of ventricular aneurysm should suggest itself.

The Master 2-step test

In the great majority of instances, in the presence of myocardial infarction, the electrocardiogram will afford a clue, if not conclusive evidence, of the presence of this lesion. In fact, it is unusual for myocardial infarction to be present, particularly if it is recent, in the absence of electrocardiographic abnormalities. However, in the presence of myocardial ischemia,* whatever the cause (but usually on the basis of coronary artery disease), it all too often happens that patients will have symptoms, usually chest discomfort or pain, with normal electrocardiograms. When this occurs, the history, the symptoms, such as location of the discomfort, its radiation, the circumstances under which the symptoms occur and are relieved, are of utmost importance in determining the diagnosis. Indeed, the diagnosis of angina pectoris or coronary insufficiency is justifiable on the basis of the history alone in the absence of abnormal electrocardiograms if the history is entirely characteristic. However, it always is important when the physician can have the assurance of the electrocardiogram to lend support to such a serious diagnosis. In order to help clarify the diagnosis in those patients who have symptoms suggestive of coronary disease, but perhaps not entirely typical, and who have normal electrocardiograms, further electrocardiographic tests have been devised. The test most commonly accepted and used and the one in which the findings are considered to be most affirmative, provided they are properly interpreted, is the Master 2-step exercise test. This test has been the most widely employed and accepted of those used to determine impairment of coronary reserve and presence of coronary insufficiency, and consequently more data concerning normal controls over a wide range of patients have been accumulated than with other exercise tests. This test is not without some danger and should not be performed on patients in whom the diagnosis is evident. It should be performed in the presence of a physician. At times the Master 2-step test is performed to evaluate response to treatment.

Even after the Master 2-step test is performed, there may remain some patients who still will have a normal response to the test and yet will have been proved eventually to have had coronary artery disease. However, these incidences are relatively few. Certainly the Master test has helped to remove from the doubtful group a large number of diagnostic problems involving chest discomfort. On the other hand, a comment of caution should be made at this point. There is no doubt that some patients have been condemned to a life of psychologic invalidism and economic stress through misinterpretation of the results of the Master 2-step test.

As with other tests, there is some dispute concerning the criteria for a diagnosis of a positive test indicative of the presence of coronary artery disease. The most significant feature of a positive 2-step test is an S-T segment depression of more than 0.5 mm. from the isoelectric level. The development of characteristic chest pain during the test is considered to be of some significance as is the development of bundle branch block or conversion of an upright T wave to a diphasic or inverted T wave in Leads I or II or the chest leads.

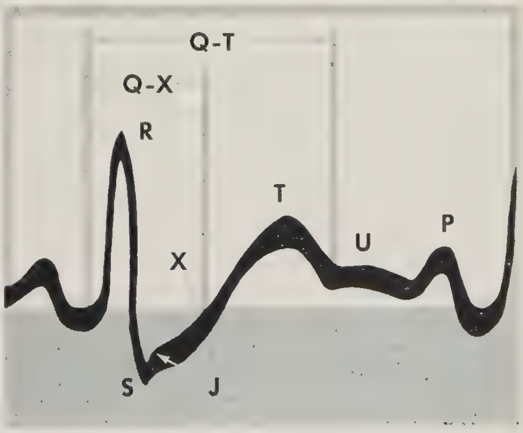

Fig. 728. Diagram by Master and Rosenfeld showing method of determining the QX/QT ratio; modification of a drawing by Lepeschkin and Surawicz: New England J. Med. 258:512, 1958. This expresses the configuration of RS-T in a quantitative manner, independently of heart rate. (From Roche Report on Medical Progress, Aug. 8, 1960.)

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*Characterized by absence of death of heart muscle tissue but the presence of insufficient oxygenation.

Recently Master[2] proposed a more refined criterion for evaluating the results of his exercise test. This criterion involved the determination of the QX/QT ratio, in which Q is the interval from the onset of QRS to X, the point at which a depressed S-T segment returns to the isoelectric line and the QT is the interval from the onset of QRS to the termination of T.

Master concluded that ratios of more than 0.5 were significant of organic heart disease. This criterion was found to be 97% accurate in the male and 83% in the female.

Technic of performing the Master 2-step test*: "The patient is prepared essentially as for a basal metabolism test. After a good night's rest, he should either have no breakfast or only a dry piece of toast or cracker and a cup of weak tea. Smoking and medicines are prohibited that morning. In order for the patient to be at ease, a matter-of-fact, calm atmosphere should prevail. There should be a short rest period just before the test is started.

"The two steps are each 9 inches high, and the subject ascends the steps and walks down the other side (Fig. 729). This is counted as one trip. He then turns and retraces his steps. This is another trip. . . .

"The method of performing the 'two-step exercise test' should not be varied. Before any exercise is undertaken, the 12-lead resting electrocardiogram must be normal. It is usually taken with the patient in a recumbent position. If a photographic electrocardiograph is used, the tracing should be developed before the test is done.

"While the exercise is being performed, the electrodes should be kept in place. The number of trips varies with the age, sex, and weight of the patient (Table 368). To give the patient a sense of security, the steps are placed near a wall. Thus, should the patient so desire, he can support himself. As each trip is completed, the subject should turn in the same direction, i.e., either always toward the wall or always toward the person supervising the test. An occasionual firm grip on the patient's arm as he climbs the steps, without exerting any upward force, may increase his confidence. A large clock or a wristwatch with a sweep second hand should be used to regulate rate of ascent and descent of the patient. If the pace is too slow, he is urged to go faster; if he walks too rapidly, he is asked to slow his rate. This is done so that the specified number of trips may be completed at the end of the time allotted for the test: 1½ minutes (single test) or twice the number of trips in 3 minutes (double test). A variation of 1 to 3 seconds is of no importance.

"When the exercise has been completed, the patient immediately returns to the recumbent position. An electrocardiogram is taken at once, another is taken 2 minutes later, and a third 6 minutes after cessation of the exercise. Originally, we preferred to record the three standard leads and one precordial lead, usually V_4 or V_5, whichever had the better combination of a tall R and small S wave. The precordial leads have been found to be the most informative, however, and for several years now we have been taking only Lead II of the limb leads (frontal plane) and precordial leads V_3, V_4, and V_5, or V_4, V_5, and V_6 (horizontal plane). These 4 leads can easily be obtained within 40 seconds. On rare occasions electrocardiographic changes may be missed if more than a minute is required for the 'immediate' record, but 1 minute is actually ample time to obtain the first postexercise electrocardiogram.

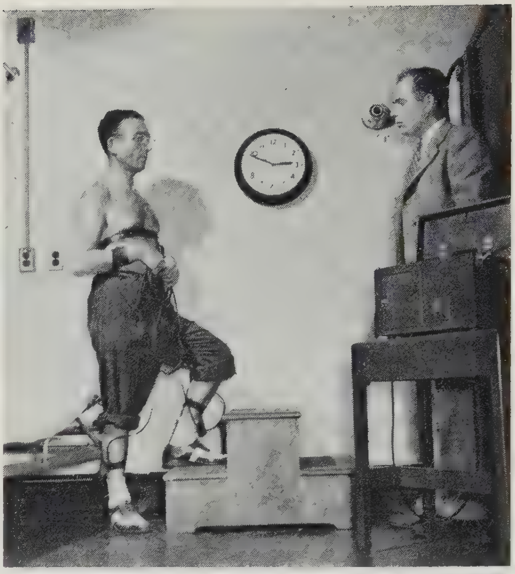

Fig. 729. Picture of steps used in Master 2-step test. (Courtesy Dr. Arthur M. Master, New York, N. Y.)

"When the base line (line of reference) of the electrocardiogram ascends, there is a tendency for the RS-T segment to be elevated artificially. It may be depressed when the base line descends. For this reason, it is wise to obtain a record of 3 or 4 beats on a horizontal level, so that the reading of the exercise electrocardiogram can be accurately made. . . ."

The proper base line (isoelectric level) from which to ascertain deviations of the S-T segment has been the subject of differences of opinion.

The T-P segment is often subject to artifactual alteration, or drifting, and when the heart rate is fast may even be absent. The P-R segment is relatively stable and its level is therefore easier to determine. It is thus the author's opinion that the level of the P-R segment should be used as the level of reference for the determination of deviations of the S-T segment. Master is likewise of the opinion that the P-R segment is the proper base line rather than the T-P segment.[4]

"Precautions: If the following simple precautions are heeded, the test is completely safe:

"1. The 'two-step test' should be performed only if the 12-lead resting electrocardiogram taken immediately prior to the test is normal.

"2. The exercise should be discontinued if the patient complains of chest pain.

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*From Master, A. M., Field, L. E., and Donoso, E.: New York State J. Med. **57**:1051, 1957.

Table 368. *"Master 2-step test," standard number of ascents, men and women, by age and weight**

Weight (lb.)	*Age (yr.)*												
	5-9	*10-14*	*15-19*	*20-24*	*25-29*	*30-34*	*35-39*	*40-44*	*45-49*	*50-54*	*55-59*	*60-64*	*65-69*
							Males						
40- 49													
50- 59	33	35	32										
60- 69	31	33	31										
70- 79	28	32	30										
80- 89	26	30	29	29	29	28	27	27	26	25	25	24	23
90- 99	24	29	28	28	28	27	27	26	25	25	24	23	22
100-109	22	27	27	28	28	27	26	25	25	24	23	22	22
110-119	20	26	26	27	27	26	25	25	24	23	23	22	21
120-129	18	24	25	26	27	26	25	24	23	23	22	21	20
130-139	16	23	24	25	26	25	24	23	23	22	21	20	20
140-149		21	23	24	25	24	24	23	22	21	20	20	19
150-159		20	22	24	25	24	23	22	21	20	20	19	18
160-169		18	21	23	24	23	22	22	21	20	19	18	18
170-179			20	22	23	23	22	21	20	19	18	18	17
180-189			19	21	23	22	21	20	19	19	18	17	16
190-199			18	20	22	21	21	20	19	18	17	16	15
200-209				19	21	21	20	19	18	17	16	16	15
210-219				18	21	20	19	18	17	17	16	15	14
220-229				17	20	20	19	18	17	16	15	14	13
							Females						
40- 49	35	35	33										
50- 59	33	33	32										
60- 69	31	32	30										
70- 79	28	30	29										
80- 89	26	28	28	28	28	27	26	24	23	22	21	21	20
90- 99	24	27	26	27	26	25	24	23	22	22	21	20	19
100-109	22	25	25	26	26	25	24	23	22	21	20	19	18
110-119	20	23	23	25	25	24	23	22	21	20	19	18	18
120-129	18	22	22	24	24	23	22	21	20	19	19	18	17
130-139	16	20	20	23	23	22	21	20	19	19	18	17	16
140-149		18	19	22	22	21	20	19	19	18	17	16	16
150-159		17	17	21	20	20	19	19	18	17	16	16	15
160-169		15	16	20	19	19	18	18	17	16	16	15	14
170-179		13	14	19	18	18	17	17	16	16	15	14	13
180-189			13	18	17	17	17	16	16	15	14	14	13
190-199			12	17	16	16	16	15	15	14	13	13	12
200-209				16	15	15	15	14	14	13	13	12	11
210-219				15	14	14	14	13	13	13	12	11	11
220-229				14	13	13	13	13	12	12	11	11	10

*From Master, A. M., Field, L. E., and Donoso, E.: New York State J. Med. 57:1051, 1957.

"3. The test should not be done when the patient is sick.

"4. A single test should always first be performed on patients. Only when the single test is negative should the double test be undertaken. The latter is usually done an hour after the single test or on the following morning.

"With these precautions we have never had an accident during the performance of the 'two-step exercise test.' So-called modifications of the test may be dangerous since they usually require excessive physical exertion. They are, in addition, of little clinical diagnostic value since no criteria have been established to determine what constitutes a positive result in these various modifications.

"**Criterion of a positive test:** An RS-T depression of more than 0.5 mm. in any lead is our criterion of a positive test. T-wave alterations may often occur with the RS-T depressions. Minor T-wave changes alone are less significant, but definite T-wave inversion is probably abnormal. Transient arrhythmias or bundle branch block are of little diagnostic importance."

In addition to establishing the diagnosis of coronary artery disease, the Master 2-step test has been found useful in detecting the presence of a deficient coronary circulation in those persons who have no cardiac symptoms but are engaged in a type of work in which the occurrence of a heart attack would endanger not only themselves but also the people dependent upon them.

Other uses have been made of this test in the evaluation of the effects of various drugs on the coronary circulation.

Fig. 730 illustrates electrocardiographic changes in a positive Master 2-step test.

Pericarditis

The electrocardiogram is often of considerable aid in the diagnosis of pericarditis both with and without effusion. This is not to say,

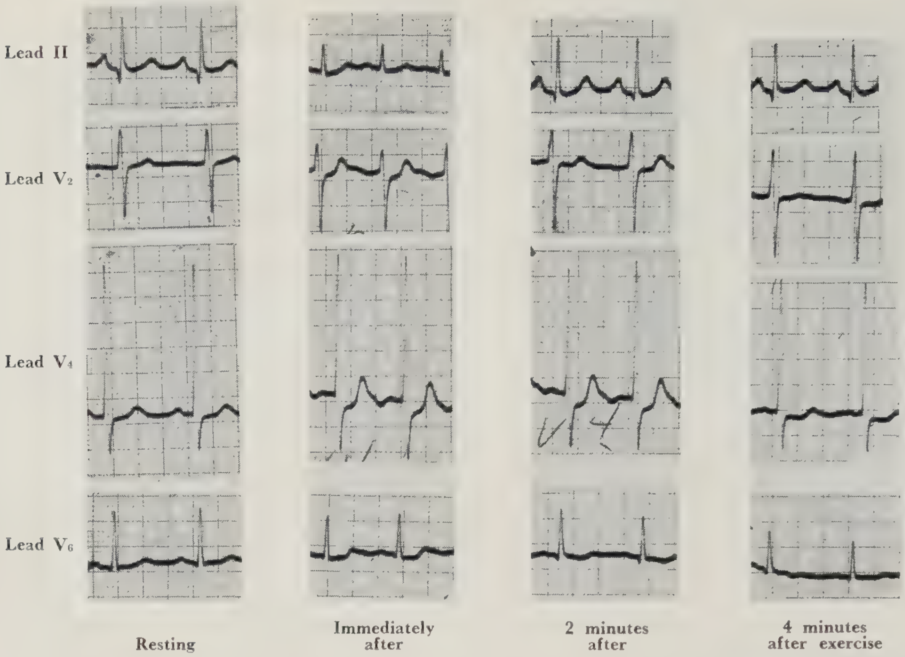

Fig. 730. Positive Master 2-step test. Note the decrease in amplitude of T_2 immediately after exercise, the depression of ST of 1 mm. in V_2 and particularly the depression of ST in V_4 of 2 mm. persisting until 4 min. after exercise, when the depression has decreased to 1 mm. These tracings are indicative of embarrassment of the coronary circulation.

however, that in this condition the electrocardiogram may not be normal. As in so many other clinical states, a normal electrocardiogram does not exclude the presence of pericarditis. Another confusing consideration is that underlying abnormalities other than pericarditis, such as myocardial ischemia, hypertension, electrolyte imbalance, etc., may obscure the electrocardiographic pattern usually associated with pericarditis.

In pericarditis there is inflammation of the pericardium and the superficial heart muscle adjacent to it. Death or necrosis of the heart muscle does not occur unless there is some other factor such as coronary thrombosis. Since significant Q waves are indicative of heart muscle death, abnormal Q waves will not be expected to occur as a result of pericarditis. This is an important difference that distinguishes the electrocardiogram of myocardial infarction from that of pericarditis. The changes most characteristic of pericarditis occur in the S-T segments and T waves. These are best elicited by taking serial electrocardiograms. The early sign is an elevation of the RS-T segment that usually occurs in all the standard leads and the chest leads. This may be a point of differentiation from myocardial infarction in which the elevation occurs in Lead I or Leads II and III with **reciprocal** depression in Leads III or II and I, depending on the site of the infarct.

The RS-T elevation progressively becomes less so that after several days the RS-T segment is at the isoelectric level, and if only one electrocardiogram were taken confined to this period, the resulting normal electrocardiogram would give no clue as to the presence of pericarditis. The T waves then progressively become lower in amplitude so that eventually they are isoelectric, diphasic, and finally inverted. Here again it is important to stress that the T-wave changes, just as is the case with the RS-T segment elevation, occur often in all the standard leads and are not reciprocal as would be the case in myocardial infarction. Subsequently, the T waves will often but not always return to normal, this period being variable but usually being several weeks. Another distinction from myocardial infarction is that the electrocardiographic changes are much more evanescent and less likely to be permanent in pericarditis.

Another striking type of electrocardiographic change is found when pericarditis has resulted in the formation of effusion. Under this circumstance there is likely to be low voltage in all the standard leads and often in the chest leads. This low voltage is caused by

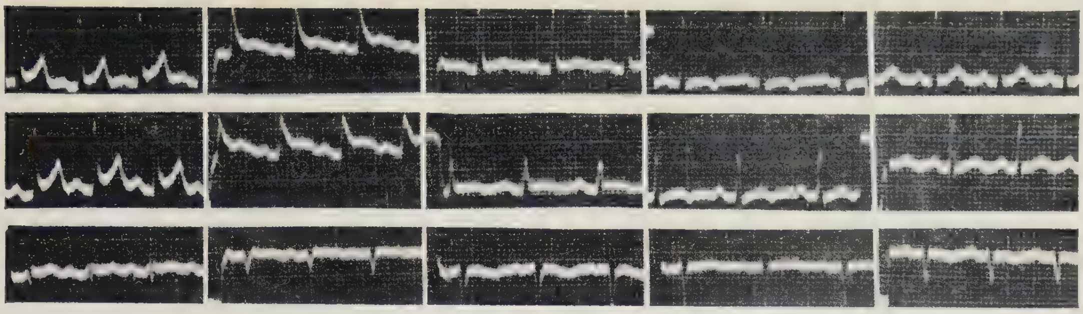

Fig. 731. A series of electrocardiograms of a patient with rheumatic pericarditis. (From Scherf, D., and Boyd, L. J.: Clinical electrocardiography, Philadelphia, 1946, J. B. Lippincott Co.)

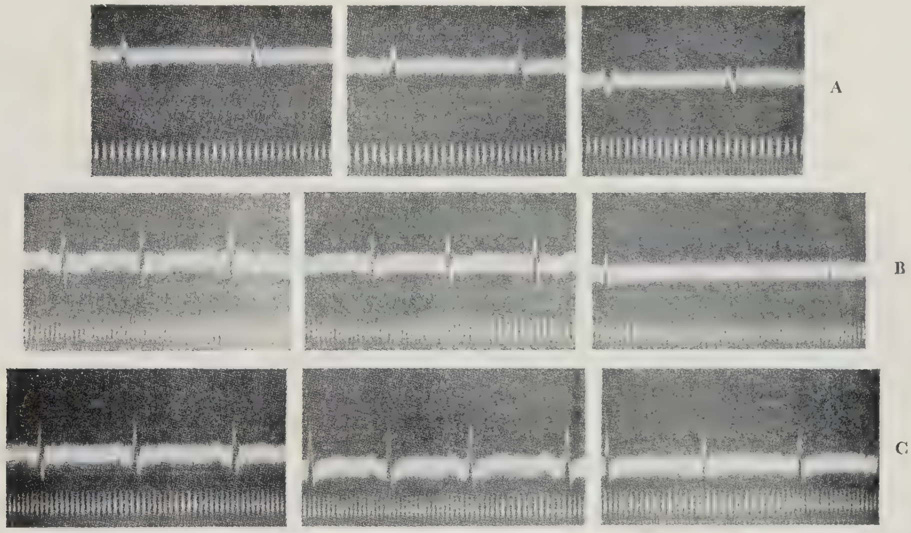

Fig. 732. A, Electrocardiograms in a pericardial effusion. **B,** After removal of 500 ml. fluid. **C,** After absorption of effusion. (From Scherf, D., and Boyd, L. J.: Clinical electrocardiography, Philadelphia, 1946, J. B. Lippincott Co.)

the short circuiting of the electrical potentials of the heart by the pericardial fluid. One must be cautious about interpreting low voltage since it may occur in normal persons (in whom the P and T waves are of normal amplitude), in myxedema, and in severe pulmonary emphysema.

If pericardial adhesions form as a result of pericarditis, the heart may be fixed in a relatively immobile position in the chest, and electrocardiograms taken with the patient in various positions will fail to show a shift in the electric axis of the electrocardiogram.

Syphilitic heart disease

The characteristic lesion of syphilitic heart disease is aortic regurgitation. Since changes in an electrocardiogram depend on muscular involvement and not on valvular defects, the effect of aortic regurgitation on the electrocardiogram will be caused by secondary results of this valve lesion, i.e., left ventricular enlargement. As stated above, increase in size of the left ventricular muscle causes left axis devitation. It is thus evident that the electrocardiographic pattern that one expects for the usual syphilitic involvement of the cardiovascular apparatus is not specific for that condition. It may occur as well in any other condition causing left ventricular enlargement. If the syphilitic aortic regurgitation is early or is only slight and no significant left ventricular enlargement has occurred, the electrocardiogram may then show a normal axis. Other changes (nonspecific) in the electrocardiogram may occur if the mouths of the coronary arteries are obstructed by a syphilitic lesion of the aorta with resultant fibrosis of the myocardium. The electrocardiographic effects will then be not unlike those of chronic coronary obstruction from other causes—inversions of T waves, alterations in conduction, spread and notching of QRS complexes, and increased P-R interval.

Acute pulmonary artery obstruction

Acute pulmonary artery obstruction is a condition that is often catastrophic and may cause death instantly or within a short period of time. The electrocardiogram often gives characteristic patterns that will fortify the physician in his decision to take heroic measures for treatment. It should be emphasized, however, that the electrocardiogram may be entirely normal in this condition.

In characteristic examples, the S wave in Lead I is prominent and wide, and leads rather abruptly and by a steplike configuration of the shortened S-T segment into the T waves. Q waves are often present in Leads II and III, and T_2 and T_3 are inverted. Note that the pattern of Q_3 and inverted T_3 is that described for posterior infarction. The prominent, wide S_1 and the characteristic S-T segment in Lead I, as well as the much more transitory nature of the picture in acute pulmonary artery obstruction, all help to differentiate it from posterior infarction in which the changes endure for months and are usually permanent. The electrocardiogram of acute pulmonary artery obstruction often though not necessarily reveals right axis deviation because of the strain on the right heart. Fig. 733 illustrates an electrocardiogram in acute massive pulmonary artery obstruction.

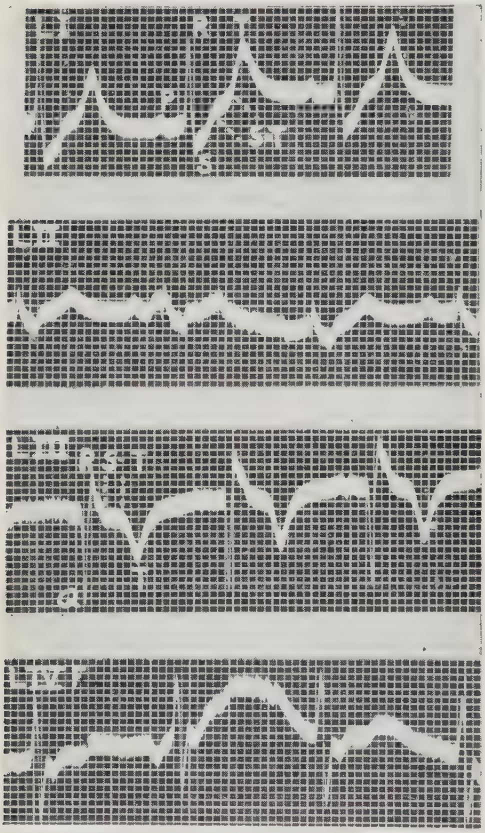

Fig. 733. Acute massive pulmonary artery obstruction. Note the prominent wide and slurred S wave and the short steplike S-T segment in Lead I. The deep Q wave and inverted T wave in Lead III resemble those of posterior wall myocardial infarction. (Cambridge spring instrument using photographic equipment.)

Pulmonary emphysema and pulmonary artery sclerosis

Pulmonary emphysema and pulmonary artery sclerosis may cause changes in the electrocardiogram by increasing the strain on the right ventricle and thus result in right axis deviation. The P wave is often characteristically altered in pulmonary emphysema. It becomes tall and sharply peaked in Leads II and III (the P pulmonale) in contrast to the P wave of mitral stenosis, which is broad, prominent, and notched in Leads I and II. The T waves not infrequently may be low or inverted. Fig. 712 shows an example of P pulmonale.

Any other conditions that affect oxygenation of the heart muscle, such as anemias, can be expected to cause lowering or inversion of T waves.

Electrolyte disturbances and the electrocardiogram

It has been well known that certain electrolyte disturbances have distinct and often recognizable effects on the electrocardiogram. The electrolytes of clinical significance that have been studied with relation to the electrocardiogram are chiefly potassium and, to a less extent, calcium. These electrolytes may cause electrocardiographic disturbance by being either increased or decreased in the blood.

Potassium is the most important of the electrolytes in this connection. It has been established that its passage in and out of the cardiac cell is vitally influenced by digitalis. In certain cardiac arrhythmias caused by digitalis intoxication, such as paroxysmal tachycardia with block, the administration of potassium is of therapeutic importance.

The changes in the electrocardiogram roughly correlate with the level of the serum potassium. In hyperpotassemia, with levels between 7 and 9 mEq./L. potassium (normal about 5 mEq./L.), there is increase in T-wave amplitude and decrease in its width (Fig. 734). As the level of potassium increases above this, the P wave disappears as a result of standstill of the atria (Fig. 735). When the potassium level reaches 10-10.5 mEq./L., progressive widening of QRS occurs until it finally fuses with the T wave (Fig. 736). At 12-14 mEq. complete cardiac cessation occurs.

In hypopotassemia there may be entirely normal electrocardiographic patterns, but in general the following changes occur:

1. S-T segment depression with lengthening of the Q-T interval.
2. Inversion of the T wave and lengthening of the Q-T interval.
3. Increase in amplitude of the T wave which remains upright with prolongation of Q-T interval.
4. Presence of a U wave with a small T wave.

The electrocardiographic patterns accompanying increased potassium levels in the blood have been used as a means of following the efficacy of therapy directed toward correcting this abnormality, although this may be difficult to evaluate since other factors such as digitalis therapy or myocardial disease may influence the T wave, S-T segments, and Q-T interval.

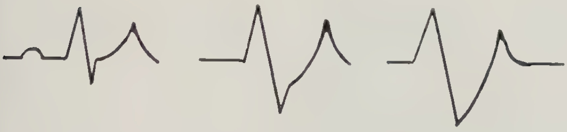

Fig. 734 Fig. 735 Fig. 736

Figs. 734-736. Effect of slight, moderate, and marked hyperkalemia on the electrocardiogram. (From Dimond, E. G.: Electrocardiography, St. Louis, 1954, The C. V. Mosby Co.)

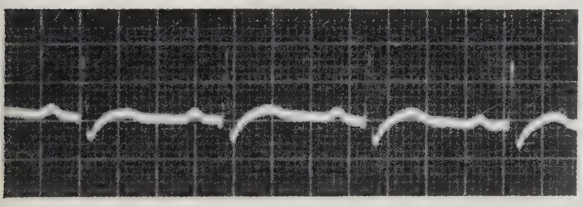

Fig. 737. Effect of increased serum calcium on electrocardiogram. Note shortened Q-T interval. (From Dimond, E. G.: Electrocardiography, St. Louis, 1954, The C. V. Mosby Co.)

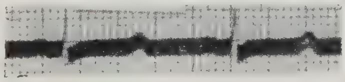

Fig. 738. Effect of decreased serum calcium on electrocardiogram. Note prolonged Q-T interval. (From Dimond, E. G.: Electrocardiography, St. Louis, 1954, The C. V. Mosby Co.)

Calcium is another electrolyte that is known to affect the electrocardiogram at times. Increased level of calcium (hypercalcemia) may cause a shortening of the Q-T interval. The T wave may be increased in amplitude (Fig. 737).

Decreased calcium levels (hypocalcemia) produce prolonged Q-T interval (Fig. 738).

Thyroid disturbances

Thyrotoxicosis is commonly associated with an electrocardiogram that shows merely normal complexes occurring at a faster than normal rate, i.e., sinus tachycardia. Occasionally the QRS and T waves may show greater voltage than usual. One common abnormality of rhythm that is manifested by hearts exhibiting the effects of the increased cardiovascular strain of thyrotoxicosis is atrial fibrillation, described below under "abnormal rhythms."

The opposite picture of thyrotoxicosis, myxedema, is often associated with electrocardiographic changes revealing low QRS and low or inverted T waves as well as slow rates (sinus bradycardia).

Deficiency diseases

Deficiency diseases, of which beriberi is an outstanding example, often show electrocardiographic evidences of myocardial involvement such as low ventricular complexes (QRS and T) and inversion of the T waves.

Malnutrition often causes prolonged Q-T interval and decrease in amplitude of all the complexes. In extreme starvation the heart rate is often decreased.

Pellagra and beriberi frequently cause the T wave to become inverted. In addition, in these states there are RS-T elevations and often low ventricular complexes as well as increased heart rate.

Toxic states

Acute toxic conditions such as diphtheria and scarlet fever may cause changes in T waves—flattening or inversion—and varying degrees of heart block—increase in P-R interval.

Chronic toxemias, such as uremia, often exhibit effects on the electrocardiogram. The QRS interval is often increased, the T waves are inverted, the S-T segment is depressed, and the Q-T interval is increased as well. Pericarditis, a complication of uremia, may superimpose its effect upon the electrocardiogram. P waves and Q-T alterations may occur in uremia secondary to the increase in serum potassium levels often seen in uremia.

Drug effects

Digitalis: It is important to consider the effects of certain drugs on the electrocardiogram. Digitalis, the most important drug used in the treatment of heart disease, exerts certain characteristic effects. The most characteristic effect is on the S-T segment which descends abruptly without any tendency toward upward convexity as in the coronary S-T segments. The T waves are also depressed and eventually may become inverted with full digitalis effect.

Digitalis also manifests an effect on atrioventricular conduction so that the P-R interval is increased to the extent that even marked degrees of heart block are produced. In toxic effects many different types of disturbances of rhythm, most often extrasystoles, are produced. Fig. 739 shows the effect of digitalis on the electrocardiogram.

Quinidine: Quinidine is a valuable drug in the treatment of certain serious cardiac

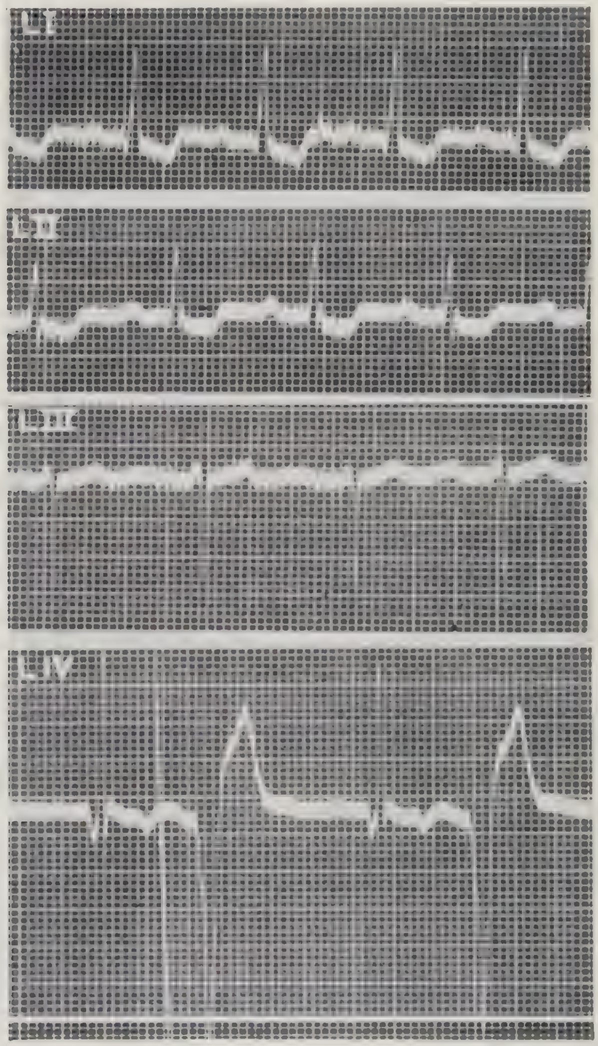

Fig. 739. Digitalis effect. The S-T segments are depressed with abruptly downward deviation and no convexity upward. The T waves have become inverted in Leads I, II, and IV. The characteristic changes are here most typically shown in Leads I and II. In Lead IV are seen ventricular premature beats occurring every second cycle (pulsus bigeminus), a toxic manifestation of digitalis. The P-R interval is increased to 0.23 sec., this being also a toxic effect of digitalis.

arrhythmias, such as paroxysmal ventricular tachycardia, paroxysmal superventricular tachycardia, and atrial flutter. However, in overdosage it can be dangerous. The use of the electrocardiograph is an important means of preventing overdosage with quinidine since quinidine causes well-defined effects on the electrocardiogram. Quinidine causes prolongation of the QRS complex, and for this reason it should not be used except with the greatest of caution in patients having intraventricular conduction defects (right and left bundle branch block and arborization block).

Furthermore, in a patient who is under intensive treatment with quinidine, electrocardiograms should be taken at frequent intervals, and if the QRS complex becomes significantly prolonged (perhaps 25% more than normal), further therapy with quinidine should be continued only after careful consideration of the calculated risks involved in continuing or stopping the administration. Quinidine, in addition to its effect on the QRS complex, causes slowing of the heart rate, prolongation of the Q-T interval, and lengthening of the P-R interval and P waves. Although it does not appear to be described in the literature, the author has seen markedly widened, large, and bizarre T waves with consequent prolonged Q-T interval often enough to consider this effect as characteristic of high quinidine dosage.

Neurocirculatory asthenia

The importance of distinguishing psychogenic effects on the electrocardiogram from those of organic diseases has been pointed out. These psychogenic effects are manifested by flattening, notching, or inversion of the T waves usually in Leads II and III or the chest leads and very occasionally in Lead I. They have often been mistaken for organic effects. The importance of distinguishing between the changes in the electrocardiogram occasionally associated with neurocirculatory asthenia and those caused by organic heart disease is obvious since it involves matters affecting the happiness of the patient—prognosis, insurability, physical activity, desirability of marriage, etc. That such distinction can often be made has been pointed out by Wendkos, who restored abnormal T waves to normal by the use of certain drugs that act upon the autonomic nervous system. This type of pattern usually occurring in the young has been referred to as the "juvenile pattern."

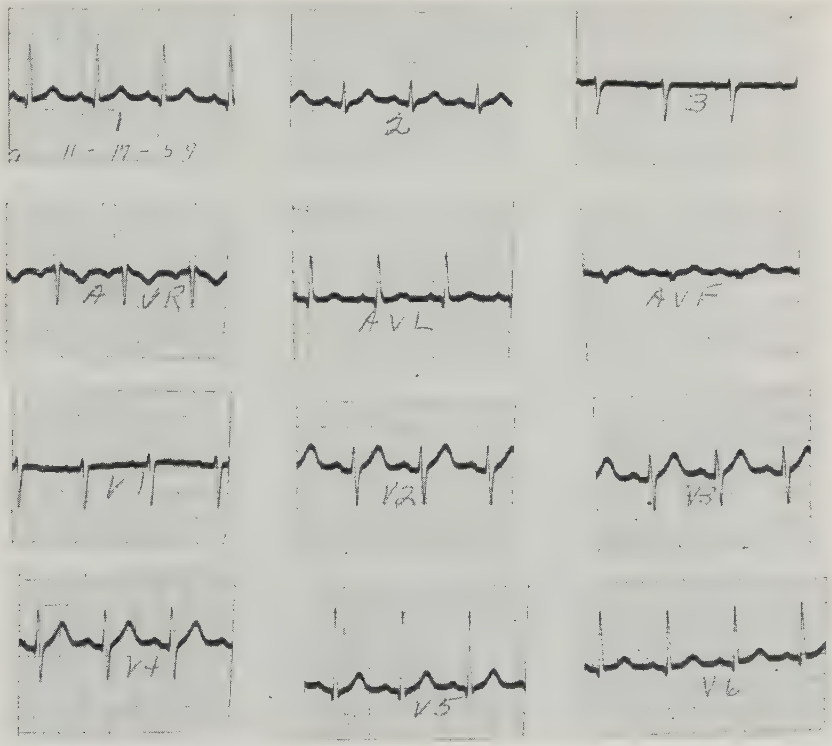

Fig. 740. Sinus tachycardia. The configuration, duration, and relationship of the complexes to each other are normal. The heart rate is 115/min.

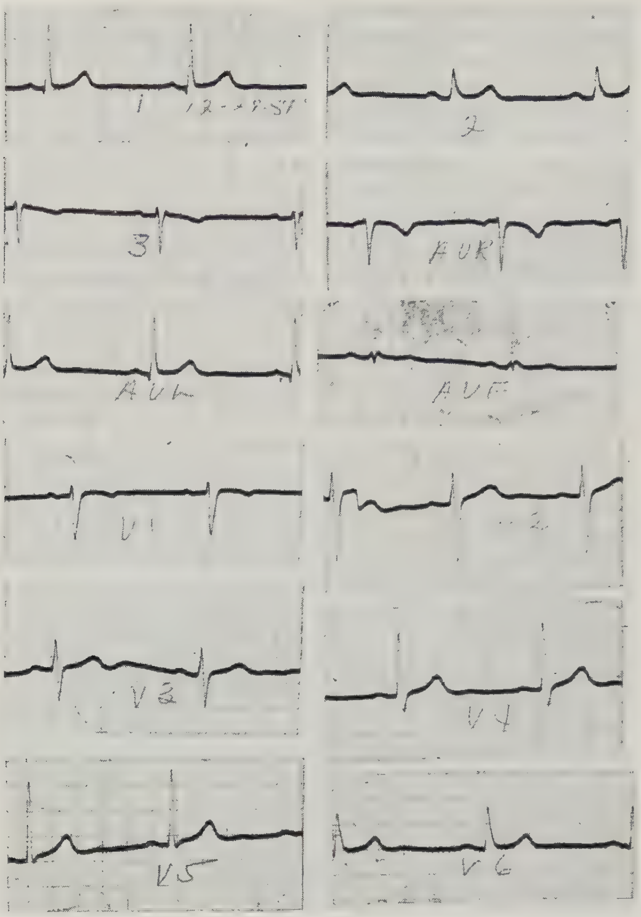

Fig. 741. Sinus bradycardia. The configuration, duration, and relationship of the complexes to each other are normal. The heart rate is 53/min.

In many patients either with or without organic heart disease, a great variety of disturbances in rate, rhythm, and conduction may occur. It is important to remember that these abnormalities in many instances do not necessarily indicate the presence of organic heart disease, but that, on the other hand, some are more likely to be associated with serious heart disease than others. It is not our purpose here to describe these clinical correlations but rather to describe briefly those disorders in mechanism that are more commonly seen.

NORMAL VARIATION IN RATE AND RHYTHM

In a normal electrocardiogram the normal sequence of complexes, P, QRS, and T occurring in their correct relationships with each other and at a rate of 60-100/min., is known as a normal sinus mechanism. The impulse starts in its normal place in the sinus node and passes through the atria down the A-V node, the bundle of His, the right and left bundle branches, and throughout the ventricles in a normal manner.

Sinus tachycardia: Impulses emanating from the sinus node occur at a rate exceeding 100/min. Because the impulse follows the normal conduction pathways, the complexes are situated normally with respect to each other. The increased rate results in varying degrees of shortening of the T-P interval. If the rate is fast enough, the T-P interval may be so shortened that P waves are superimposed upon the preceding T waves. Fig. 740 shows sinus tachycardia.

Sinus bradycardia: Here again the mechanism of conduction is normal, and consequently the relationships of the complexes are normal. The rate, however, is below 60/min. Fig. 741 shows sinus bradycardia.

Sinus arrhythmia: The impulse for cardiac contraction originates in the normal place in the sinus node. However, because of variations in vagus nerve tone affecting the speed of impulse formation in the sinus node, the rhythm varies, with regularly recurring faster and slower cycles. The configuration of the complexes and the relationships between the atrial and the ventricular complexes are normal; the only variation is a regularly recurring increase and decrease of the T-P intervals with the slower and faster cycles. This mechanism is considered a normal alteration in rhythm. It depends, as a rule, upon the phase of respiration, an increase in rate occurring with inspiration, and a decrease with expiration.

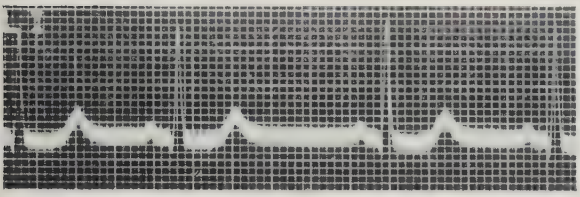

Fig. 742. Sinus arrhythmia. Note that the atrial and ventricular complexes are of normal configuration and have normal time relationships. The only alteration is the shortening and lengthening of the T-P intervals.

ABNORMAL VARIATIONS IN MECHANISM

Disturbances in pacemaker

Since the impulse that originates cardiac contraction normally starts in the sinus node, this node is known as the pacemaker. The site of origin may shift from its normal place and occur anywhere along the conduction system from the atria to the ventricles. This abnormal disturbance in site of the pacemaker may occur as a result of functional or nervous influences, of toxic effects such as those due to drugs, and of organic disease involving the conduction system.

Sinus arrest (sinoatrial block)

In this mechanism there is a dropping out of a complete atrial cycle together with its

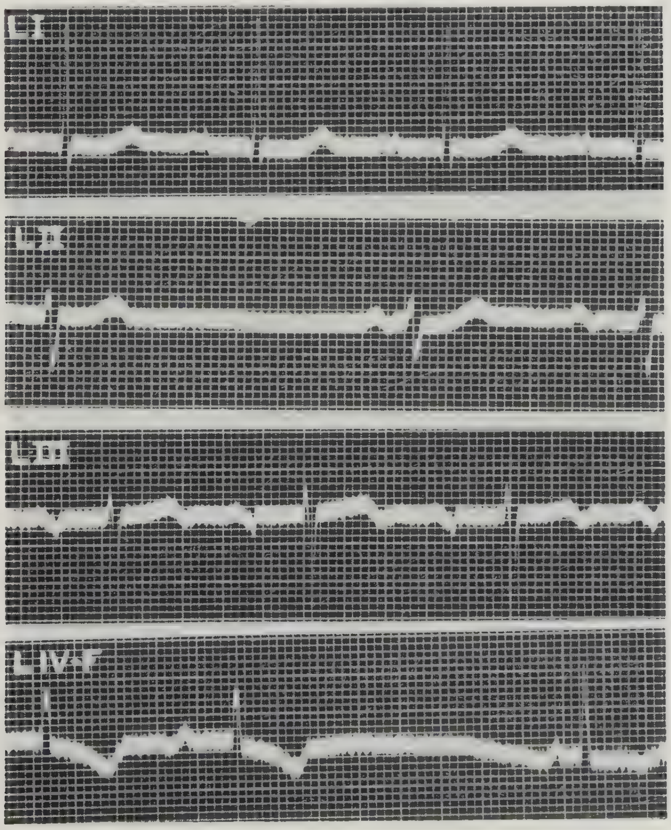

Fig. 743. Sinus arrest (sinoatrial block), also delayed A-V conduction. Note dropping out of a complete heart cycle including P and QRS and T waves in Lead II.

accompanying ventricular cycle, resulting in a pause usually almost the length of 2 normal cycles. The impulse at the sinus node has either not occurred at all or it has been blocked from reaching the atrial muscle. Fig. 743 shows sinoatrial block.

Ventricular escape

In ventricular escape the impulse emanates in the bundle of His or on rare occasions below the bifurcation of the bundle. This occasionally occurs in sinus arrest when there is depression of the higher centers of impulse formation with rising degree of irritability of the lower centers, to a point at which the lower center "escapes" from the control of the higher center (sinus node) and originates an impulse of its own. In this pattern a ventricular complex is seen after a long pause. This complex is normal because the impulse is supraventricular in origin, but no P wave precedes it. If, as rarely happens, the focus originates below the bifurcation of the bundle, the form of the ventricular complex becomes

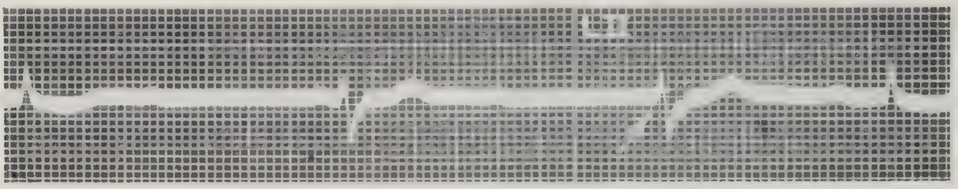

Fig. 744. Ventricular escape. Note that the "escaping" ventricular complex is not preceded by a P wave and is of similar configuration to the preceding one except for the slight distortion of the S limb due to a superimposed P wave. The electrocardiogram also shows 2:1 heart block.

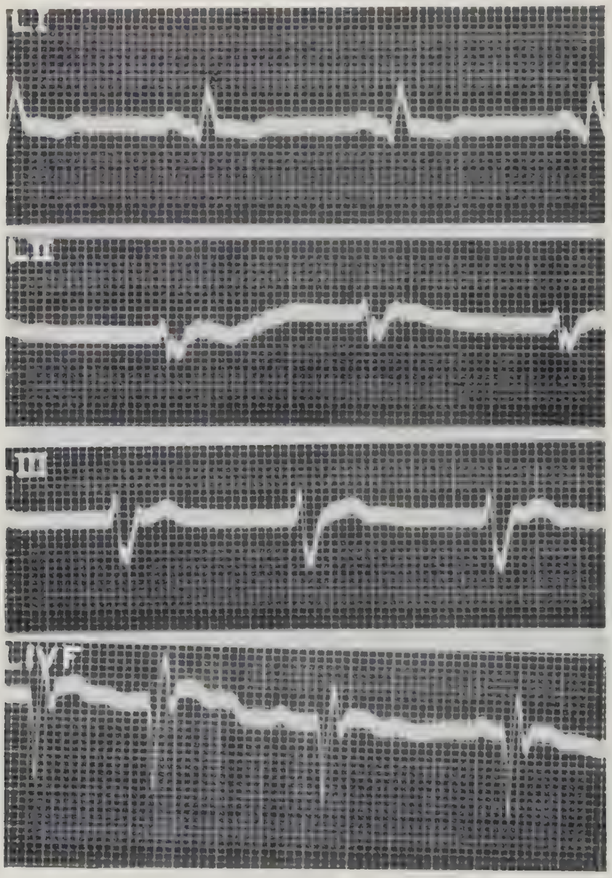

Fig. 745. Shifting pacemaker. In Leads I and IV F, the time relationship of P to QRS varies, with slight variation in form of P especially evident in Lead IV F.

distorted (aberrant). Fig. 744 shows ventricular escape.

Shifting pacemaker

In this mechanism the site of impulse formation may wander anywhere from the sinoatrial node to the atrioventricular node. The farther the impulse occurs from its normal origin in the sinoatrial node, the greater the alteration in the P wave. These alterations vary from only minor changes in the shape of the P wave to inversion of this wave. The relationship of P to QRS changes so that the P may precede the QRS by a much shortened P-R interval or the P may be buried in or may even follow the QRS. Contour of the QRS does not change since the site of impulse formation is supraventricular. There is sometimes slight arrhythmia, as may be expected. See Fig. 745.

Nodal rhythm

Here the site of pacemaking impulse formation is in the atrioventricular (A-V) node. Since this is a supraventricular site, the ventricular complexes are of normal contour. The rhythm is regular. The P wave may not be seen; it may be buried in the QRS or it may occur immediately before or after the QRS, depending on whether the impulse reaches the atria or the ventricles first. See Fig. 746.

Premature contractions (extrasystoles)

The existing rhythm may at times be interrupted by a contraction of the heart caused by an irritable focus that may arise elsewhere than in the sinus node—in the atria, in the A-V node, in the bundle of His, or in the right or left ventricle. These contractions occur early after the normal cycle, some time before the next normal contraction is ordinarily expected to appear. The site of origin of these contractions can be detected by the characteristic alterations of the electrocardiographic complexes and their time relationships. Premature contractions are usually followed by a pause which, in the case of atrial extrasystoles, is longer than the normal pause between 2 cycles but is not equal to the sum of 2 cycles. The pause following extrasystoles arising in the ventricles, is **compensatory;** that is, the length of the 2 cycles is equal to 2 normal cycles. There is an exception to this. Occasionally a ventricular extrasystole occurs without interrupting the regularity of the rhythm. No compensatory pause occurs, and in this case the extrasystolic beat may be followed by a pause shorter than the normal interval. Such ventricular extrasystoles are termed **interpolated.**

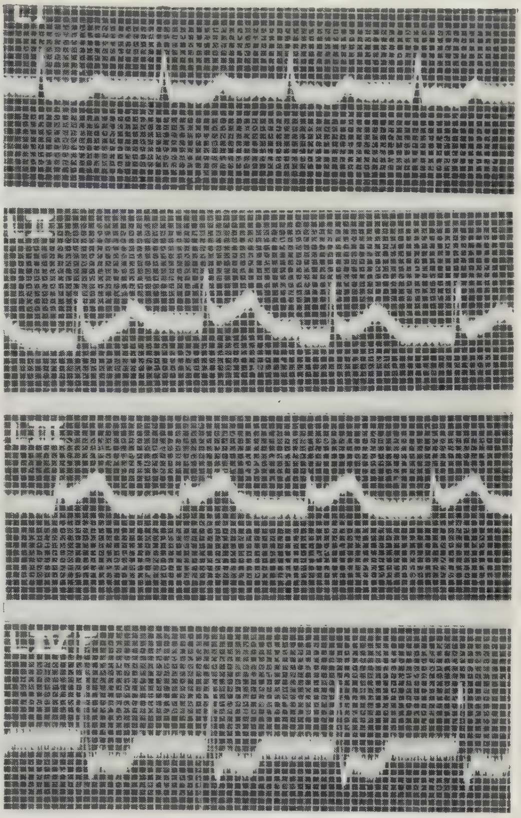

Fig. 746. Nodal rhythm. The rhythm is regular and the rate is 100. The P waves cannot be seen and are buried in the QRS complexes. The ventricular complexes are supraventricular in form. The impulse has probably originated in the middle of the A-V node. If it had originated in the lower portion of the node, P waves would be visible following the QRS and would be of abnormal contour; if it had originated in the upper portion of the A-V node, the P would have preceded QRS with a very short P-R interval. In all these types, P waves may be absent. Note that the ventricular complexes in this electrocardiogram indicate that posterior wall myocardial infarction is present.

The greater the distance from the normal site that the premature focus arises, the greater is the change in appearance of the extrasystolic complex from that of the usual one for the patient.

Premature systoles may occur with varying frequency. The rhythm is termed **bigeminy** when they occur every second cycle, **trigeminy** if they occur every third cycle, and **quadrigeminy** if they occur every fourth cycle. It is possible to see numerous extrasystoles of variable origin in the same electrocardiogram.

Premature atrial systole (atrial extrasystole): When an irritable focus in the atrium other than the sinoatrial node gives rise to an

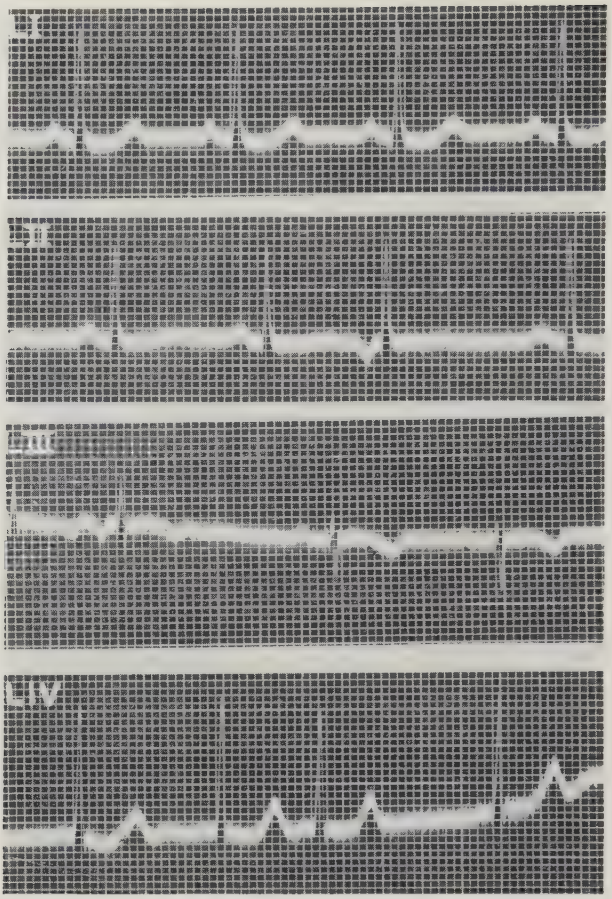

Fig. 747. Premature atrial contraction. The premature beat comes earlier than anticipated. The P waves of the premature cycles in Leads II, III, and IV are inverted and are followed by ventricular complexes of normal configuration except in Lead III where the QRS and T are slightly altered (aberrant). The P-R interval of the regular cycle in Lead II is 0.15 sec.; that of the extrasystole is 0.11 sec. Note that the pause following the premature atrial contraction is not compensatory.

atrial contraction, a premature atrial systole results. It is characterized by a P wave that occurs before it is expected, sometimes superimposed upon the preceding T wave, but differing in form from the usual P waves for that particular record. This alteration in form may vary from only a slight degree to a marked degree characterized by a completely inverted P wave, depending upon the distance between the point of origin of the premature focus and the sinoatrial node. The P-R interval is increased or decreased and the QRS and T waves are unaltered in form, although these may occasionally be changed (aberrant). At times an extrasystolic P may occur without a corresponding QRS; that is, the ventricles may be refractory and do not respond to the extrasystolic impulse from the atrium, or the extrasystolic impulse from the atrium may be entirely blocked from reaching the ventricles. The pause that follows an atrial extrasystole is greater than the interval between 2 normal cycles, but the total time between the cycle preceding the premature systole and the one following it is less than the total of 2 normal cycles. In other words, the pause following a premature atrial systole is not usually compensatory.

Premature nodal contractions (nodal extrasystoles): The impulse for this type of premature contraction arises in the atrioventricular node or the bundle of His. This impulse travels downward to the ventricle and backward to the atrium and produces both an atrial and a ventricular contraction. If the backward-traveling impulse reaches the atrium at the time of the ventricular contraction, the P wave is concealed in the QRS complex. The P waves, when visible, are inverted and may occur before or after the QRS, depending on when the impulse has reached the atria in relation to the time that the opposite traveling impulse has reached the ventricle. Since the impulse has reached the ventricles from above (supraventricular), the form of the ventricular complexes will not be altered, or it will be altered only to a slight degree (slightly aberrant). The pause following this type of premature contraction may or may not be compensatory.

In rare instances the retrograde impulse does not reach the atria, or the atria are unresponsive (refractory) to the backward traveling impulse. The atria in such cases continue to respond to the normally traveling impulses from the sinoatrial node, and the P waves are normal in shape and direction. See Fig. 748 illustrating premature nodal systole.

Premature ventricular contraction (ventricular extrasystole): In this type of premature contraction the focus arises either in the right or in the left ventricle below the bifurcation of the bundle of His. The ventricular complexes occur before the next regular complex. The QRS complex is characterized by increased width, notching, and slurring and usually increased amplitude. The accompanying T is usually in a direction opposite to the main deflection of the QRS; it is of greater amplitude and width than the other T waves.

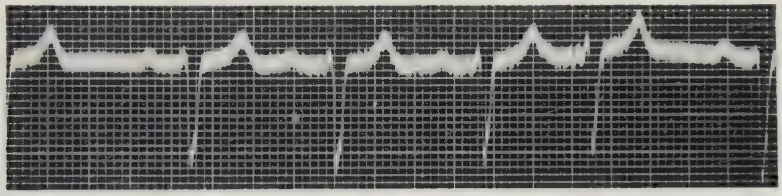

Fig. 748. Premature nodal contraction (Lead III). The premature contraction occurs early. The ventricular waves of the nodal contraction are normal in configuration (supraventricular). The P wave is not visible.

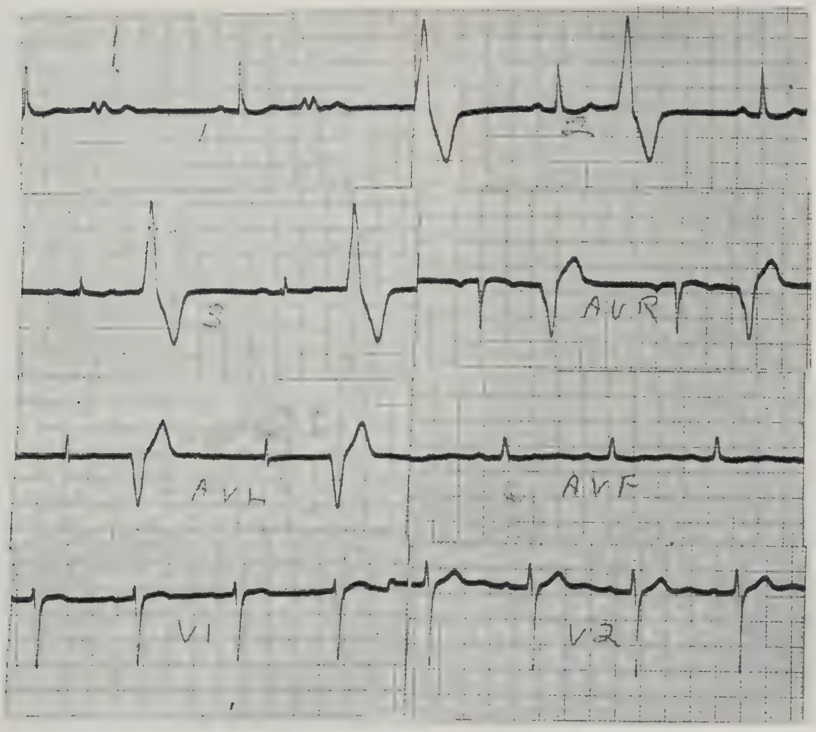

Fig. 749. Ventricular premature contraction (extrasystole). The QRS complexes are widened and slurred, and the T waves are large and in opposite direction from the main deflection of QRS. The premature ventricular complexes occur every second cycle in Leads I, II, III, $\mathrm{aV_R}$, and $\mathrm{aV_L}$ (pulsus bigeminus).

The pause that follows the ventricular extrasystole is compensatory; that is, the sum of the duration of the preceding cycle and of the cycle following the extrasystole is equal to the duration of 2 normal cycles.

Atrial rhythm is not influenced, but the P waves are often lost in the premature ventricular complexes. The ventricles are refractory shortly after the extrasystolic contraction; thus they do not respond to atrial stimulus during this phase. Sometimes the refractory phase has passed before it is time for the next regular contraction. In such cases no dropping out of the regular cycle is evident, and the extrasystole is followed by a pause that is less than the interval between 2 successive normal cycles of the record. The extrasystole is then termed **interpolated.**

If the premature focus arises in the left ventricle, the main deflection of QRS is down in Lead I (Fig. 749); if it arises in the right ventricle, the main deflection is up in Lead I. The main deflections of QRS in Leads II and III may be up or down in either left or right ventricular extrasystoles, although usually an upward main deflection of QRS in these leads indicates a left ventricular extrasystole and a downward main deflection indicates a right ventricular extrasystole.

Paroxysmal tachycardia

Paroxysmal tachycardia is a term applied to a series of rapidly recurring heart cycles that have their origin in an abnormal focus in the atria, in the atrioventricular node or bundle, or in the ventricles. These episodes are characterized by the suddenness with which they start and stop; hence the term

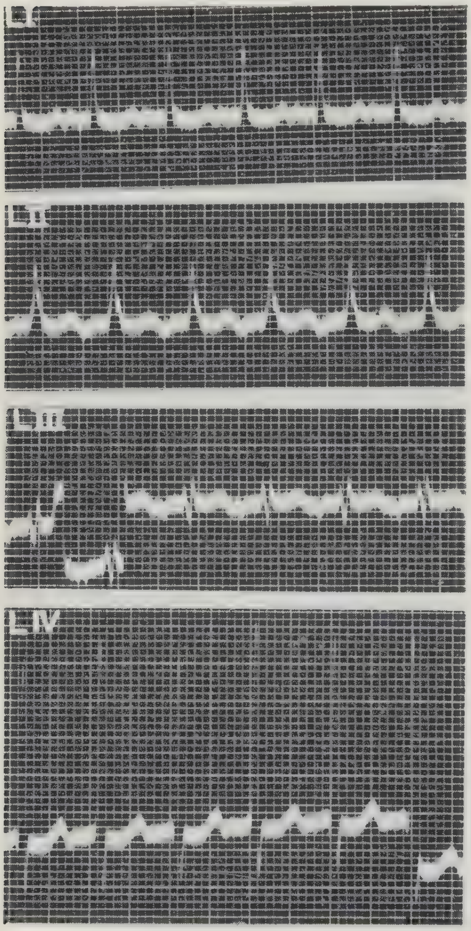

Fig. 750. Paroxysmal atrial tachycardia. The rate is 157 and the rhythm is regular. The P waves in Lead I are tiny and follow immediately the preceding T waves. In Leads II and III the P waves are inverted. The P-R interval is 0.12 sec. The QRS is of normal duration and of normal configuration.

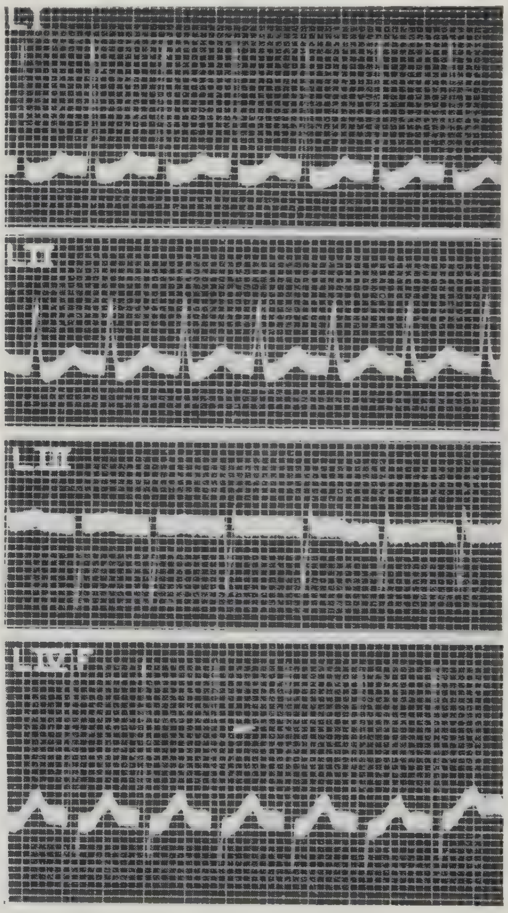

Fig. 751. Paroxysmal nodal tachycardia. The appearance of the ventricular complexes is supraventricular and like that in nodal rhythm. However, the rate is much faster—171/min. The rhythm is perfectly regular. The P waves, as in the example of nodal rhythm, cannot be identified.

"paroxysmal." They may last from a minute or more to several days. The rate usually is quite constant, depending upon the origin of the impulse formation. The rate may be between 150 and 250/min. Because they originate in some abnormal irritable focus, these tachycardias may be regarded as a series of rapidly recurring premature systoles. These tachycardias are termed paroxysmal atrial, nodal, or ventricular tachycardia, deriving their names from the site of the abnormal focus.

Paroxysmal atrial tachycardia: This type of mechanism is thought of as a series of rapidly recurring atrial extrasystoles. The P waves are different in form or in direction from P waves of a normal mechanism. The preceding T wave is often superimposed on the P wave so that identification of the P wave may be difficult or even impossible. In such instances differentiation of paroxysmal atrial tachycardia from paroxysmal nodal tachycardia cannot always be made. The QRS complexes are not usually altered in form, but they may be aberrant to varying degrees. Rate and rhythm are characteristically constant. Fig. 750 shows an example of paroxysmal atrial tachycardia.

Paroxysmal nodal tachycardia: Here, too, rate and rhythm are absolutely constant. The P waves are usually hidden in the QRS complex, but occasionally they occur before or after the QRS. Configuration of the QRS and T waves is not disturbed. The picture is one of nodal rhythm except for the rates which are much higher in paroxysmal nodal tachycardia. Fig. 751 shows an example of paroxysmal nodal tachycardia.

Paroxysmal ventricular tachycardia: The rate in paroxysmal ventricular tachycardia, as in the other tachycardias, may be from 150 to 250/min. The appearance is that of a series of rapidly recurring ventricular extrasystoles. The P waves are usually not identifiable, but, if seen, their rhythm is not altered. The rhythm is usually regular, although this type of tachycardia differs from nodal and atrial tachycardia in that the rhythm is often slightly irregular. This slight irregularity may at times offer a means of distinguishing atrial from ventricular tachycardia without the aid of the electrocardiograph. Fig. 752 is an example of paroxysmal ventricular tachycardia. This type of mechanism imposes a more serious prognosis, and its recognition is an urgent signal to the physician for prompt measures to terminate it.

Atrial flutter

This mechanism may be persistent or transitory. The sinoatrial node loses control of the dominant rhythm of the heart. The impulse travels a pathway about a ring of atrial musculature in a continuous cycle. This has been described as **circus movement.**

The electrocardiogram shows the atrial ac-

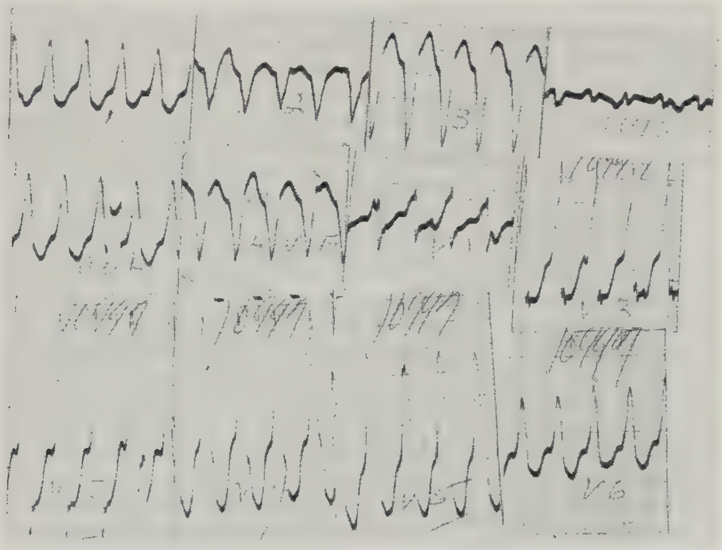

Fig. 752. Paroxysmal ventricular tachycardia. The ventricular rate is 200. The QRS complexes are widened, 0.12 sec. in duration, slurred, and notched, with T waves in opposite direction from the main deflection of QRS. The rhythm is regular.

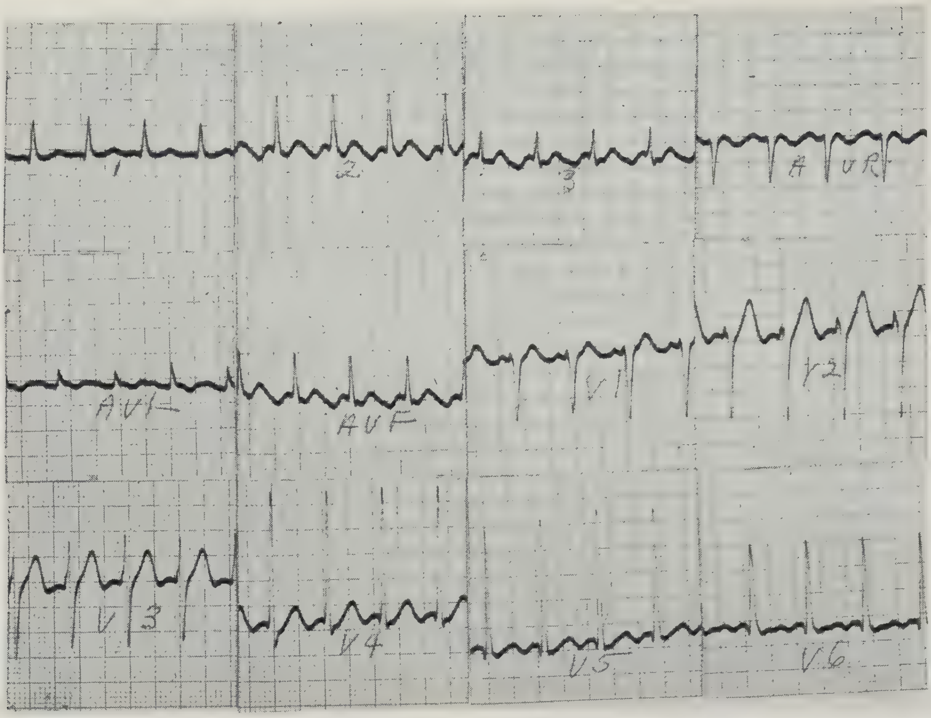

Fig. 753. Atrial flutter. The flutter waves appear as more or less regularly recurring, sawtooth deflections, most prominent in Leads II, III, and aV_F. There is a varying rate of ventricular response, from 2:1 to 3:1. The ventricular rate is 133/min. The atrial rate is 300/min.

tivity as regularly recurring sawtooth oscillations (flutter waves), interrupted by ventricular complexes at regular intervals in response to every second, third, or fourth atrial flutter waves. The form of the ventricular complexes is not altered except by the superimposition of the flutter waves themselves. The frequency of response by the ventricles to the flutter waves may vary in the same electrocardiogram so that the rhythm of the ventricular complexes may become irregular. See Fig. 753.

Rarely, a ventricular complex follows each atrial flutter wave (1:1 ratio). The flutter waves occur at rates of 200-350/min. They are most clearly identified in Leads II and III. Not infrequently they cannot be seen at all or they are much less obvious in Lead I. Fig. 753 illustrates atrial flutter.

Atrial fibrillation

Circus movements are also present in atrial fibrillation, but the pathway is shorter and irregular in its course by comparison with atrial flutter. This results in rapidly and irregularly recurring impulses that pass in an uncoodinated manner through the atrial muscle fibers. This is revealed in the electrocardiogram by absence of P waves as such. The P waves are replaced by small waves that are totally irregular in rhythm, size, and shape. These are termed fibrillary or "f" waves. These waves may at times be absent. They occur at rates of 350-500/min., but the rate is never constant. The ventricular complexes are not altered in form from the usual form for that patient. They occur in a completely irregular rhythm except in those rare instances in which there is complete blocking of the impulses from the atria to the ventricles. The less the degree of block in the bundle of His, the faster and more irregularly do the ventricular complexes occur. Atrial fibrillation may be permanent or paroxysmal. Fig. 754 illustrates atrial fibrillation.

Ventricular fibrillation

Ventricular fibrillation is characterized by the complete loss of the normal characteristics of the ventricular complex. The waves have lost their regularly recurring consistent pattern and are bizarre. They are usually large and wide, vary in size and rhythm, and altogether suggest chaos in the electrical activity of the ventricular muscle. No T waves and usually no P waves can be identified. This is invari-

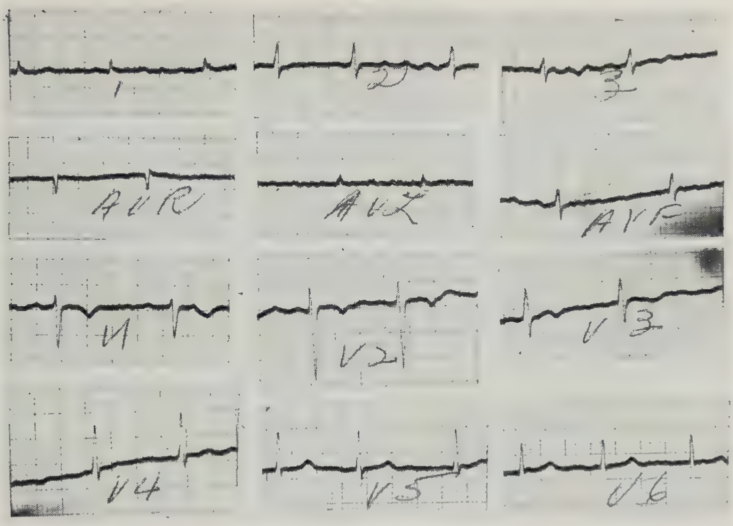

Fig. 754. Atrial fibrillation. The rhythm of the QRS-T complexes is totally irregular. The P waves are absent but are replaced by small, irregularly recurring waves that are unequal in amplitude and contour. These are the fibrillary waves, and they are best seen in Leads II and III.

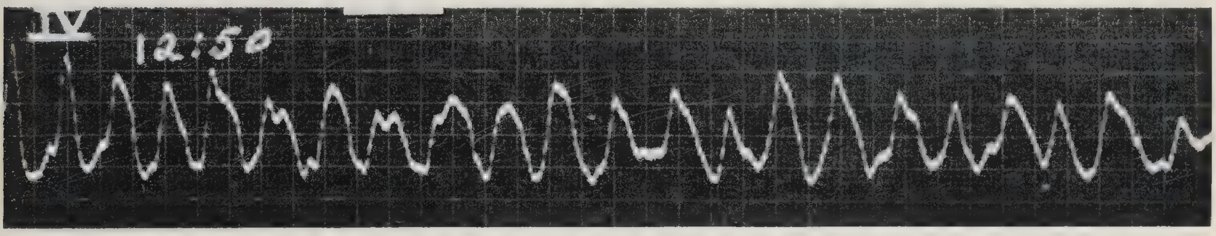

Fig. 755. Ventricular fibrillation. (From Dimond, E. G.: Electrocardiography, St. Louis, 1954, The C. V. Mosby Co.)

ably associated with death unless, as rarely happens, the ventricles can be "defibrillated" and an orderly sequence of electrical events can be restored. See Fig. 755.

Bundle branch block

When conduction of the impulse is completely blocked in either the right or the left branches of the bundle of His, a characteristic electrocardiographic pattern results. The QRS duration is increased to more than 0.12 sec. The deflections of the QRS are notched or slurred and are of large amplitude. The T wave is usually opposite in direction to the chief deflection of QRS and is of increased size. This is termed "bundle branch block." Bundle branch block may be incomplete or complete.

Incomplete bundle branch block: In this situation there is a delay rather than a complete block of conduction through one of the branches of the bundle of His. The tracings resemble complete bundle branch block except that the QRS duration is shorter than 0.12 sec.

It may be seen in both right and left ventricular hypertrophy, myocardial infarction, congenital heart disease, and pulmonary embolism in which there is right ventricular strain.

Left bundle branch block: In left bundle branch block the chief deflection of QRS is up in Lead I and usually down (discordant) in Lead III. See Fig. 756. Occasionally the chief deflection of QRS is also up in Lead III; this is then known as the concordant type of left bundle branch. The term "chief deflection" refers to the deflection of greatest duration or spread and is not necessarily the deflection of the greatest amplitude. The S-T segment is usually depressed in Lead I.

In the chest leads the characteristics are as follows: Over the left precordium the QRS complex is widened to 0.12 sec. or more. The onset of the downward stroke of the QRS complex (the intrinsicoid deflection) is de-

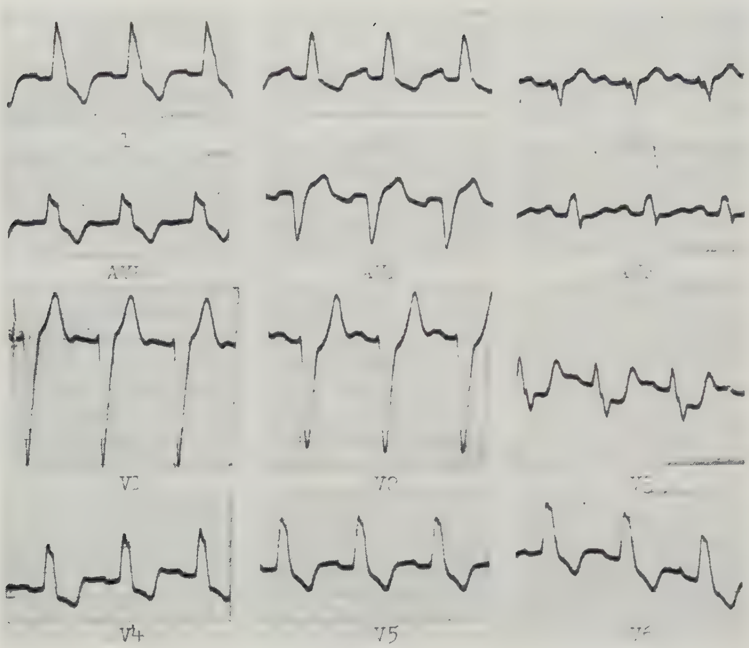

Fig. 756. Left bundle branch block (common type of bundle branch block). The QRS complex in Lead I is upright, slurred, notched, and widened with a duration of 0.12 sec. In Lead III the main deflection of QRS is down, slurred, and notched. The S-T segment in Lead I is depressed and in Lead III it is elevated. The T wave in Lead I is inverted, whereas in Lead III it is upright. In the left precordial lead (V_6) the Q wave is absent, the QRS complex being monophasic, upright, wide, and notched. The duration of QRS in Lead V_6 is 0.14 sec.

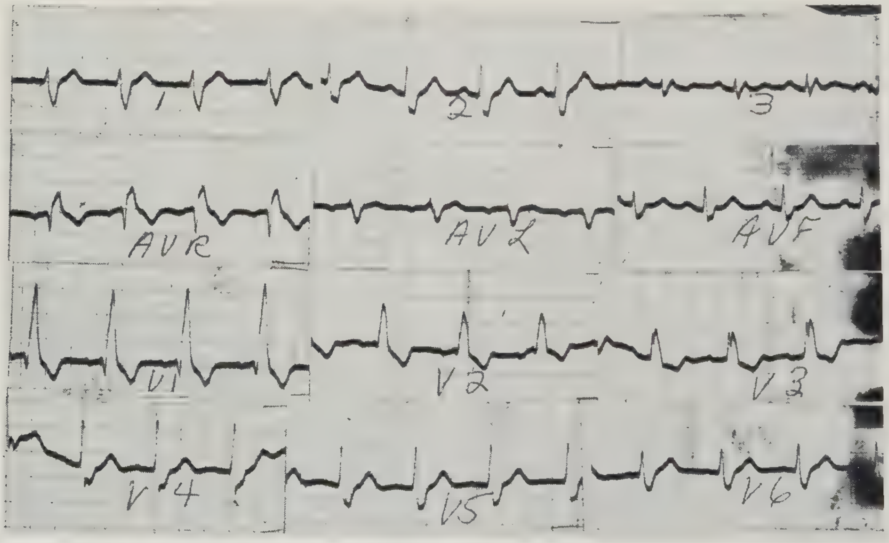

Fig. 757. Right bundle branch block. Duration of QRS is 0.13 sec. In Lead I, the R wave is relatively small and short in duration. The S wave is wide and notched and of relatively large amplitude. In Lead III there is rsr. In Lead aV_R the R′ forms a main deflection and is wide, slurred, and notched. In Lead aV_F, note that the S is wide, slurred, and notched. Most characteristically in Lead V_1, R is minute, S is small, and R′ is tall, wide, and slurred. In Lead V_6, as in Lead I, the S is wide, slurred, and notched, whereas the R is of normal duration and is relatively quickly inscribed.

layed to as much as 0.1 sec. or even more. The QRS amplitude is usually greater than normal. The S-T segment is depressed, and the T wave is usually inverted. An important feature is that the Q wave in Lead V_6 is absent, although this may not be true if infarction involves the septum itself or if there is marked counterclockwise rotation of the electrical axis of the heart.

Over the right precordium the R wave is small and is inscribed quickly (normal duration), or it may be absent. The S wave is slurred and widened. The T wave is upright, and the S-T segment may be elevated. These characteristics may be modified by variations in the electrical axis of the heart.

Left bundle branch block is seen more often than is right bundle branch block, and it is usually significant of serious organic heart disease, although it may occur in individuals otherwise considered to be normal.

Right bundle branch block: In right bundle branch block the chief deflection of QRS is directed downward in Lead I and usually upward in Lead III (Fig. 757). Another type of right bundle branch block occurs in which the R is tall and narrow and is followed by a shallow but widened S wave in Lead I.

In the right chest leads there is rsR′ configuration.* The time interval from the onset of the R to its apex and the beginning of the downward stroke is delayed from 0.04 to 0.08 sec. and is more prolonged in complete right bundle branch block as compared with incomplete right bundle branch block. The S wave in the left precordial lead is broad and slurred, but the R is normal in duration and the T is upright. The onset of R is early and of the R′ is late in the right precordial leads. Not infrequently a small R′ deflection in the right precordial leads may occur normally and may be considered to be right bundle branch block. Therefore the clinical significance of right bundle branch block must be interpreted with great caution.

Arborization block: In certain hearts there may be lesions not so much of the bundle branches as of the Purkinje fibers of the ventricles. This is sufficient to cause an abnormal widening and slurring of the QRS complex, but the QRS pattern does not have the other characteristics of either right or left bundle branch block. This represents a delay in intraventricular conduction and is termed "arborization block."

Peri-infarction block: This occurs usually in the presence of advanced heart muscle disease, usually myocardial infarction, and is characterized by widened QRS complex, a prominent Q wave, and a delayed wide R in leads in which Q is deepest. This comes about and is called "peri-infarction block" because the infarcted area cannot conduct the impulse and it must go around the infarct, thus taking a longer time to reach the epicardial muscle (Fig. 758).

Wolff-Parkinson-White syndrome

This is an infrequently seen type of electrocardiographic picture which bears a superficial resemblance to bundle branch block. It is characterized by a **shortened P-R interval** in contrast to the other types of bundle branch block in which the P-R interval is normal (or lengthened when a lesion involves the bundle of His as well as the bundle branch). The QRS is only slightly widened, 0.1-0.12 sec., and is relatively slightly notched and slurred. The most characteristic feature is a sloping and slurring of the initial ascending portion of the R wave. This is known as the "delta" wave (Δ). The RS-T segment and T are usually though not necessarily directed opposite to the direction of R. The diagnosis of Wolff-Parkinson-White syndrome is made only by means of the electrocardiograph.

That the patterns of the Wolff-Parkinson-White syndrome may vary widely has been pointed out by Burch and Kimball.[5] These authors classified the variations in these patterns into 5 groups, ranging from QRS complexes that closely resembled those of complete left bundle branch block to those QRS complexes that were of normal duration in all the standard leads. However, the shortened P-R interval was present in all these groups and gave the clue to the true nature of the condition. The author[6] has encountered a rare instance in which the type of Wolff-Parkinson-White syndrome characterized by shortened P-R interval and slurred basal portion of QRS complex with normal duration reverted spontaneously to normal patterns and then back to the Wolff-Parkinson-White patterns during the taking of the electrocardiogram.

The Wolff-Parkinson-White syndrome is not ordinarily associated with organic heart disease. However, patients who exhibit this

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*Small letters refer to the small amplitude deflections of the QRS complex, whereas the capital letters refer to the large amplitude deflections. The designation prime (′) is used to refer to a second upward (R′) or downward (S′) deflection in the QRS complex.

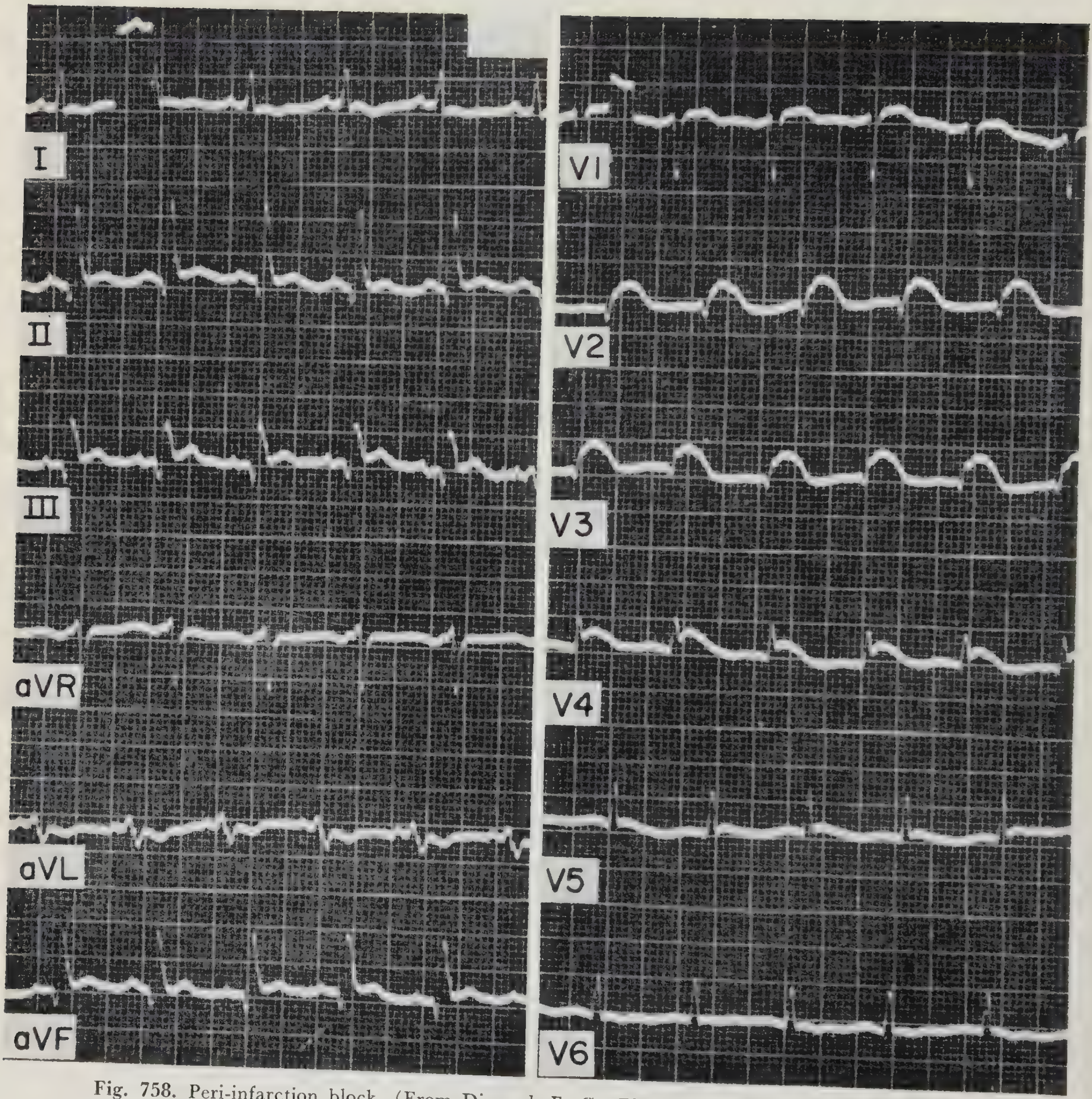
I
II
III
aVR
aVL
aVF
V1
V2
V3
V4
V5
V6

Fig. 758. Peri-infarction block. (From Dimond, E. G.: Electrocardiography, St. Louis, 1954, The C. V. Mosby Co.)

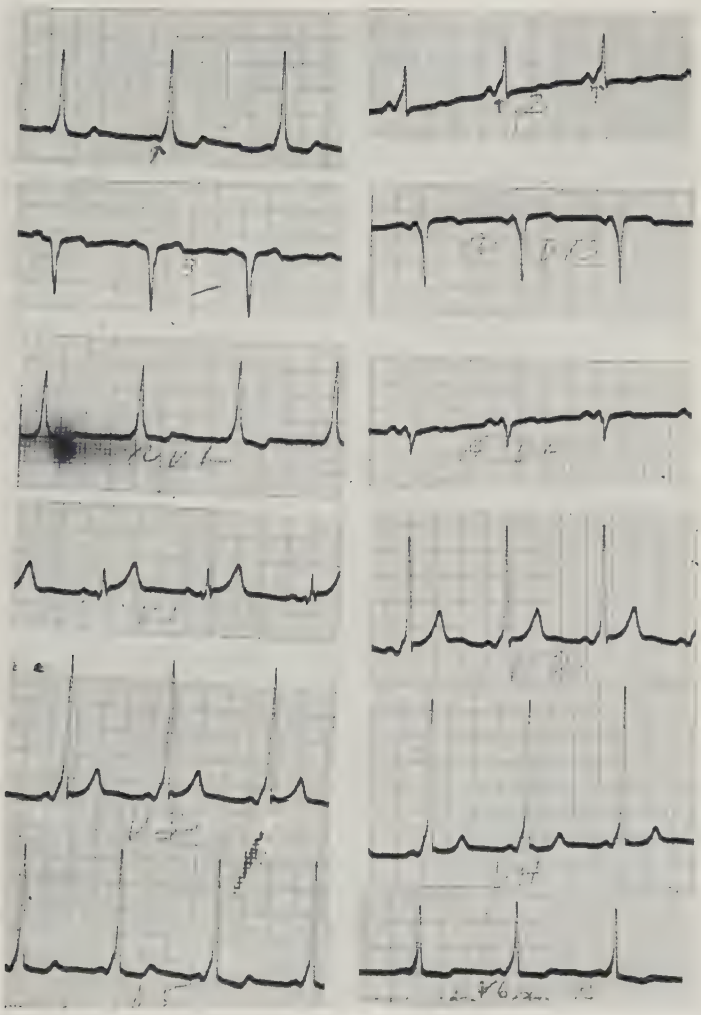

Fig. 759. Wolff-Parkinson-White syndrome. Note the short P-R interval (0.08 sec. in Lead II) and the characteristic diagnostic delta wave at the onset of the ascending limb of R, as indicated by the arrow.

electrocardiographic manifestation are likely to have episodes of paroxysmal tachycardia that on rare occasions have resulted in sudden death. Fig. 759 is an example of this interesting type of electrocardiographic pattern.

Atrioventricular block

Mention was made, in discussing the effects of rheumatic fever on the heart, of the involvement of the junctional tissues, either at the A-V node or at the bundle of His, by disease processes. Not only rheumatic fever but other types of disease as well can inhibit conduction of the impulse in the junctional tissues. These are principally degenerative diseases, drugs such as digitalis, and neurogenic influences.

First degree heart block: Degree of interference with conduction varies widely. In the milder forms there is only a slight delay in the speed of conduction so that the impulse reaches the ventricle after a greater than normal interval. The rhythm of the heart is regular, with each QRS complex following each P wave, but with a P-R interval greater than 0.2 or 0.21 sec. This degree of heart block is termed "first degree" heart block and is often referred to as "delayed atrioventricular (A-V) conduction." An electrocardiogram is invaluable in ascertaining its presence, since it is usually impossible to recognize it clinically. Fig. 760 illustrates first degree heart block.

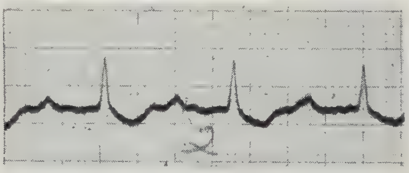

Fig. 760. Delayed A-V conduction (first degree heart block). Each atrial complex (P) is followed by a ventricular complex (QRS-T) at an interval greater than the normal of 0.2 sec.—in this instance, 0.32 sec.

Second degree heart block: As interference in conduction of impulses from the atria to the ventricles increases, some of the impulses may be completely blocked from reaching the ventricles so that the ventricles do not respond to certain of the atrial impulses, and ventricular complexes are dropped. These dropped beats may occur at infrequent intervals or as often as every other cycle. Frequently the P-R interval becomes steadily longer until eventually a ventricular complex is dropped. The P-R interval then again becomes relatively short, then progressively longer, until another ventricular complex is dropped. This is known as the "**Wenckebach phenomenon.**" Fig. 761 illustrates second degree heart block and the Wenckebach phenomenon.

Third degree heart block (complete heart block): In this type of atrioventricular block, conduction in the atrioventricular pathway is so markedly inhibited that all the impulses are prevented from reaching the ventricles. At the onset of this mechanism there is complete standstill of the ventricles for a variable length of time, ranging from a very few seconds to as much as a minute or even longer. Then the ventricles respond to their own pacemaker just below the area of blocking, either in the lower part of the bundle of His or in the ventricles themselves.

It is during this period of ventricular standstill that patients are prone to lose consciousness (**Adams-Stokes syndrome**). If the pause is longer than about 3 min., death is likely to occur.

With the response of the ventricles to their own independent pacemaker, the ventricles contract at a much slower rate than formerly, usually 30-40 times/min., or slower, and with a regular rhythm. The atria continue to contract in response to their own pacemaker in the sinoatrial node but at a much faster rate than the ventricles. Thus in the electrocardiogram the P waves and the ventricular com-

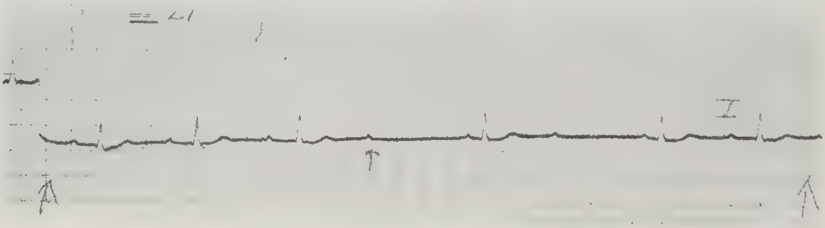

Fig. 761. Second degree heart block (partial A-V block with dropped ventricular complexes). This electrocardiogram shows that the dropped ventricular complex is preceded by progressively increasing P-R interval (Wenckebach phenomenon). Arrow indicates atrial contraction without its corresponding ventricular response.

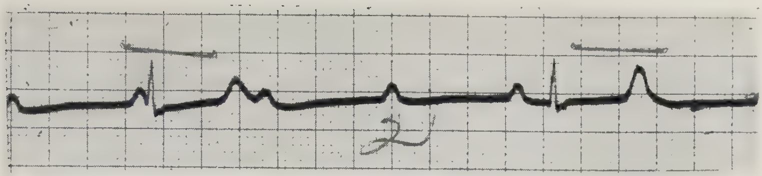

Fig. 762. Third degree heart block (complete A-V block). There is no fixed relationship between the P and QRS complexes. The atrial rhythm is regular at a rate of 92 and the ventricular rhythm is likewise regular at a rate of 30.

plexes will show no constant relationship with each other.

The form of the complexes will not vary from the usual form for the patient in heart block of any degree, since the manner of spread of the impulse through both the atria and ventricles is normal. If the complete heart block has been caused by digitalis or is congenital in origin, the ventricular rates are likely to be faster than 30-40/min. Fig. 762 is an example of complete heart block.

UNUSUAL DISTURBANCES OF MECHANISM

Pararrhythmias

Pararrhythmias represent a form of "double rhythm" in which 2 pacemakers, one of which is usually the S-A node, are active. The secondary pacemaker may be in the atria, the A-V node, or the bundle or its branches, and it may produce impulses slower or faster than those of the sinus node.

There are 2 forms of double rhythm: A-V dissociation and parasystole.

In A-V dissociation the rhythm of the atria is directed by the S-A node and that of the ventricles by the A-V node. The A-V nodal impulse has no effect on the S-A node, whereas the S-A nodal impulses do spread to the A-V node. When the S-A nodal impulse arrives at the A-V node, this causes a transitory disturbance in the rhythm set by the A-V node, provided that at that instant the A-V node and the conduction system below it are not in a refractory state. This type of rhythmic disturbance is called "dissociation with interference" (Fig. 763).

In parasystole the impulses arising in the S-A node are dominant. However, the second impulse maker, usually in the ventricle, is not influenced by the impulses from the S-A node and produces its own rhythm at a slower rate and thus one sees separate rhythms—one produced by the S-A node and the other by a regularly recurring impulse arising somewhere in the ventricles. (Fig. 764.)

The degree of disturbance of the rhythm in both these instances depends upon the relative rates of the 2 pacemakers and the refractory period of the conduction system and the ventricular muscle.

A-V dissociation may be confused with A-V heart block. The fundamental distinction is that in A-V heart block there is **depression** of conduction of varying degree in the conducting system, whereas in A-V dissociation the conduction system is normal but the S-A impulse fails to affect the ventricle because it has arrived at the ventricle during its normal refractory period.

The clinical significance depends on the cause. It may occasionally occur in an otherwise normal heart or during inflammation of the heart from rheumatic fever, toxic causes, or as a result of digitalis or quinidine therapy. The treatment depends on the cause. Both these interesting and unusual types of rhythms are not significant symptomatically and are detectable only by means of the electrocardiograph.

CONCLUSION

It must be emphasized that this section is intended to give the reader only an introduction to the subject of electrocardiography and its clinical and laboratory applications. The usefulness of the electrocardiograph in clinical medicine is established beyond question. This usefulness can, however, be impaired if one fails to recognize the many pitfalls in interpretation, such as the wide variation in normal patterns, changes due to artifacts and to faulty technic, and improper standardization of the instrument. It must be remembered that the electrocardiogram reflects changes in the myocardium which affect its electrical conductivity whatever the cause may be, and that these changes may be due to a great variety of causes, either organic or nonorganic. Such widely different causes as autonomic nervous system imbalance, position of the patient during the electrocardiographic

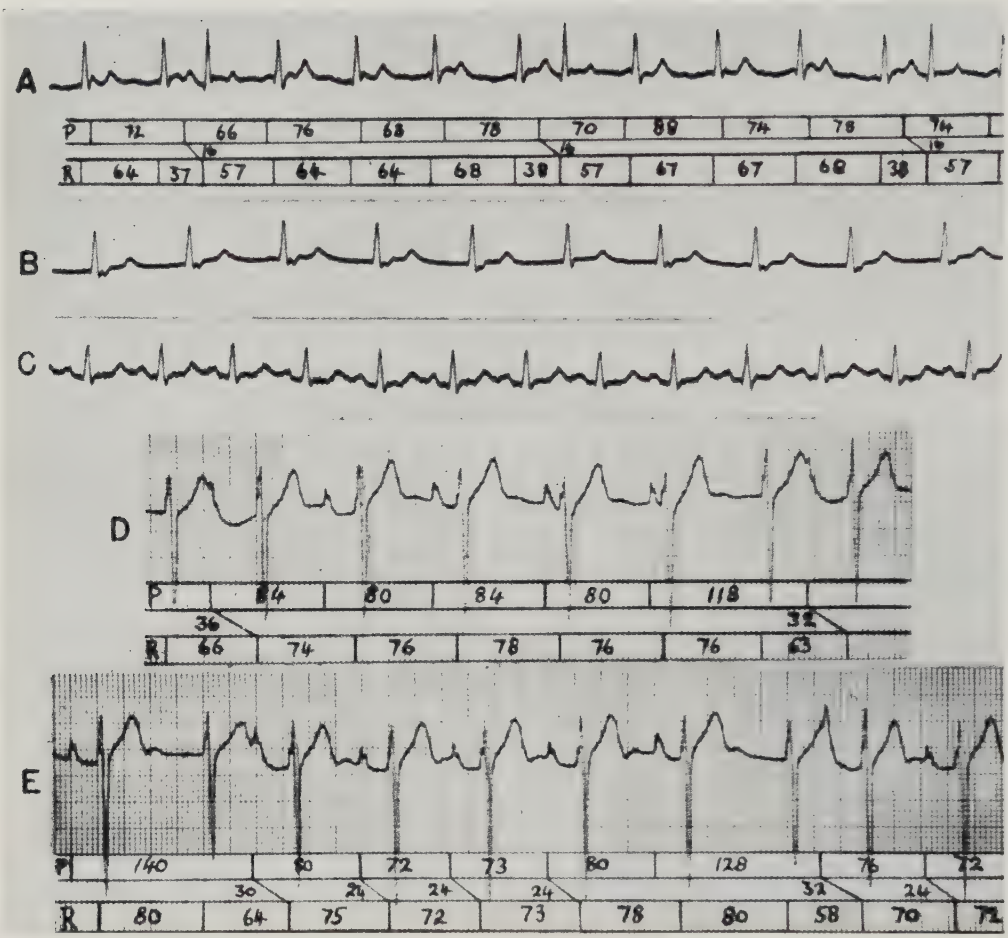
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Fig. 763. Lead II. **A,** March 27, 1957: A-V dissociation with interference; third, eighth, and last beats, which are premature, are conducted. **B,** March 29: nodal rhythm with retrograde conduction. **C,** April 1: regular sinus rhythm. Man 21 years of age, surgical repair of atrial septal defect under hypothermia. March 26: atrial flutter during operation, 1 mg. digoxin (Lanoxin) injected with no further digitalization. **D,** A-V dissociation with interference secondary to sinus arrhythmia and bradycardia, latent heart block. Bipolar right atrial lead from second right intercostal space and fourth left intercostal space on sternal border with double amplification shows upright P waves throughout. Subject is at rest; first and last P waves following A-V nodal beats are conducted, other beats are dissociated. **E,** After exercise; the sinus node is in command for 4 successive beats; P-R interval of conducted beats in late diastole shortens from 0.34 to 0.24 sec. Man 21 years of age, atrial septal defect and syncopal attacks; the latter subsided after A-V nodal automatism became established. (From Papp, C.: Double rhythms [pararrhythmias]. In Luisada, A. A.: Cardiology, vol. 3, New York, 1959, Blakiston Division, McGraw-Hill Book Co., Inc.)

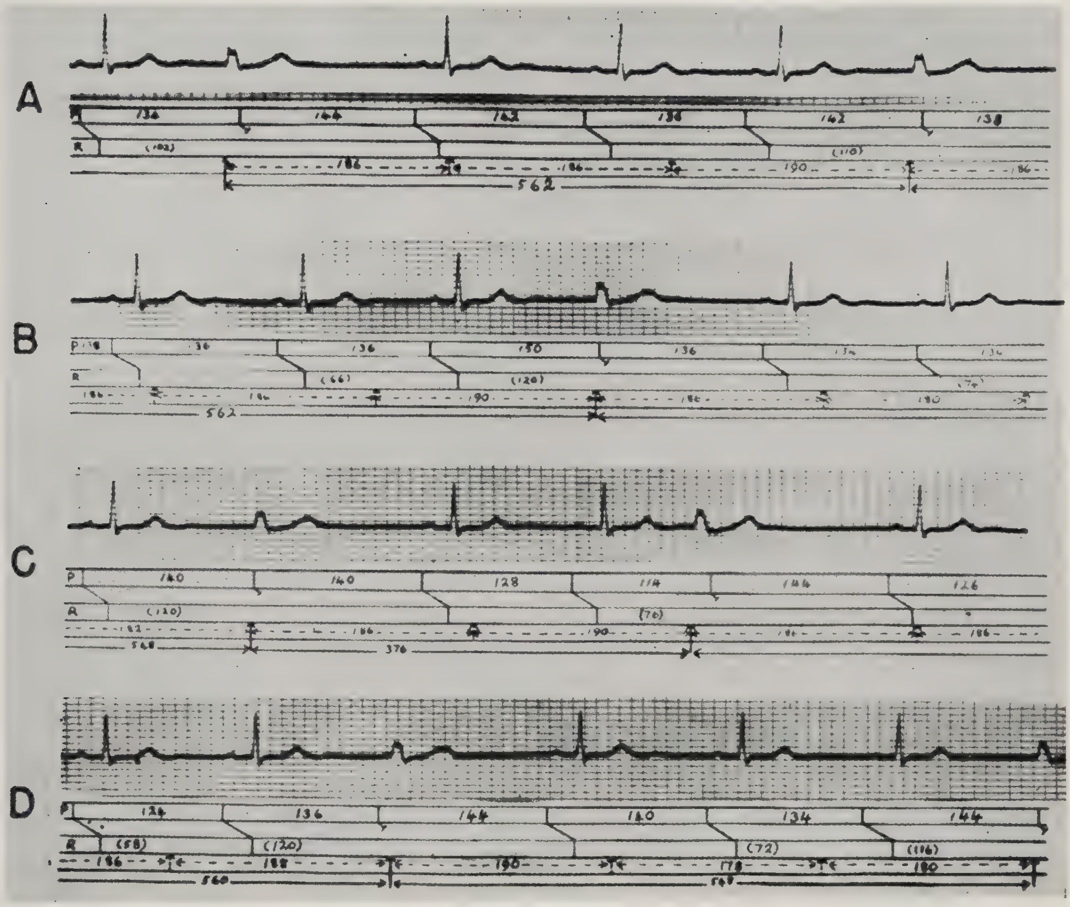

Fig. 764. Ventricular parasystole. Continuous strip in Lead II. Sinus rate, 43; rate of ectopic pacemaker, 34. Shortest coupling of manifest ectopic beats (full vertical arrows), 0.76 sec. All ectopic beats falling short of this distance remain latent (interrupted vertical arrows). Horizontal interrupted lines, distances between ectopic impulses; horizontal full lines, distances between manifest ectopic impulses. Woman, 53 years of age; coronary disease. (From Papp, C.: Double rhythms [pararrhythmias]. In Luisada, A. A.: Cardiology, vol. 3, New York, 1959, Blakiston Division, McGraw-Hill Book Co., Inc.)

recording, and actual degenerative changes in the myocardium may result in quite similar effects on the electrocardiogram. It is only after thorough familiarity with the theories and principles underlying electrocardiography and after considerable experience in its clinical application and recognition of its limitations that one can realize the full usefulness of this instrument. As previously indicated, in only a relatively few instances does the electrocardiogram give pathognomonic information, and of these instances acute myocardial infarction is an outstanding example. Many arrhythmias and conduction defects can be recognized only by the use of the electrocardiogram; these may be due to a wide variety of diseases or to functional causes. For correct clinical deductions from the electrocardiogram, the electrocardiographer should have as full a knowledge as possible of the clinical aspects of the patient's condition. It must again be emphasized that a normal electrocardiogram does not necessarily indicate the absence of heart disease; on the other hand the presence of a definitely abnormal electrocardiogram does not necessarily indicate the presence of organic heart disease. All electrocardiographic findings must be interpreted in the light of the clinical picture.

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PART VIII

Selected special topics

Chapter **113** Model of a clinical laboratory in a nonuniversity-affiliated general hospital
114 The hair in police investigation
115 Solutions for the preservation of museum specimens
116 Semen appraisal
117 Chemical analysis of water and milk
118 Skin tests

Chapter 113

Model of a clinical laboratory in a nonuniversity-affiliated general hospital

W. HAROLD CIVIN

The present-day demands on a laboratory in a large private general hospital unaffiliated with a medical school may be as great and as varied as those on a nosocomium connected with a university facility. These demands depend upon the makeup and interests of the professional staff of the hospital. Even routine laboratory work becomes more complex as medicine progresses.

If, as is now so commonplace, the professional staff is a progressive one, demands for tests are forthcoming as soon as the printer's ink describing them is barely dry. The laboratory may then be faced with the choice of refusing to perform a test while admitting its inability to do so or using a procedure that (for the laboratory in question) is as yet untried. One solution is to farm out such procedures, and another is to work out the procedures and adapt them as rapidly as possible to laboratory routine.

We have adopted the philosophy that our laboratory should function as an independent ancillary unit as far as possible and perform all feasible tests that are ordered. The laboratory being described is in a general hospital of 400 beds with a training program for internes and residents. A large expanse of water separates the area wherein the hospital is located from any undergraduate or postgraduate medical facility. This latter status may be remedied in the near future according to a number of observers on the local and national scenes. The laboratory has the full cooperation, when required, of the State Board of Health Laboratory, the State Blood Bank Laboratory, and a large military hospital laboratory. Despite this, our location and my philosophy have combined to establish the machinery to develop as nearly as possible a self-sufficient unit for performance of laboratory procedures.

Not only is our unit steered toward autonomy but it is also directed toward efficiency. To accomplish this we must deal with the factor of acquiring trained personnel. We have a chronic shortage of registered technologists, and its severity waxes and wanes. No laboratory is any better than the technologists it hires. It is important that unwise so-called economy not be allowed to run rampant to the extent of utilizing inefficient, untrained, or careless personnel because they work more cheaply. The patient suffers directly from such a pseudoeconomy, and when the attending physicians become acquainted with this state of affairs, the hospital suffers. Furthermore one must not follow in the footsteps of the legendary efficiency expert who wanted to reduce the size of symphony orchestras since various instruments were silent for varying periods of time. Laboratory technologists must not be gauged by the amount of time they spend on their feet. They are professional personnel and should be allowed to perform as such. Stresses and strains take their toll, and more efficiency is elicited by allowing technologists to pace themselves. As professionals they should naturally feel a responsibility toward the patient and a certain compulsion toward accurate performance. If they do not, they can by no stretch of the imagination be considered real technologists.

We do not use house staff members for performing laboratory procedures because we believe that this is unfair to the patient since the occasional "test performer" cannot be properly cognizant of many of his errors. We do encourage house staff members, however, to ask questions concerning procedures and results, to witness performance of tests, and even to spend 6 mo. or so in the clinical laboratories.

Because of our shortage of technologists we train some of the more capable nonprofessional employees to do certain laboratory procedures and add to their knowledge as their capabilities permit. They, however, are carefully supervised by the registered technologists.

The laboratory functions 24 hr. a day, in keeping with my philosophy. The laboratory and to some extent the technologists are departmentalized (sectionalized). Each department or section is in a separate room. This occurred originally because the wing housing the laboratory was used for surgery, and the physical divisions were already present when we moved in. The wing is air-conditioned. It is our belief that the separation of sections into individual rooms in a laboratory our size is of value. True, the pathologist cannot keep constant watch on the various sections, but members of a profession should not require foremen. The technologist is given leeway commensurate with his or her occupational status. Our technologic departmental chiefs serve to answer questions, establish procedures, and consult with the chief technologist who also acts as a coordinator of departments, orders equipment, and arranges schedules. We believe that policing technologists is not a duty for supervisory personnel nor a job for the pathologists. The technologist should have been adequately screened prior to being hired and during any probationary period. The compartmentation has been helpful to morale by promoting self-respect and self-confidence.

The departmentalization as far as technologists are concerned exists in our so-called regular or 7:00 A.M.-3:30 P.M. shift. It is during this shift that most of the routine examinations are performed. It requires an explanation to practicing physicians that the more experienced technologists are on this shift. The physicians are informed that if a great deal of routine work is done by the smaller afternoon or night shifts, emergencies are unable to be handled. It might be worthwhile here to elaborate on "stats." These are the bane of laboratory existence. In our hospital, custom maintains that technologists obtain the blood specimens for tests. This often requires a long trip from one end of the hospital to the other and back and interrupts the other work of the technologists. Quite often an explanation to the practicing physician by one of the pathologists can result in an agreement to get the result on the chart by a certain time. An occasional breakdown of this latter occurs unless the messenger service is well established. This is not always so. Phoning results may be the answer if adequate personnel is available. Pneumatic tubes although expensive are timesaving and nerve-sparing. One answer to blood drawing would be the use of ward personnel, including nurses and even house staff, plus good messenger service. For this, good rapport is needed with all departments of the hospital.

The technologists on the afternoon and night shifts are jills (or jacks) of all trades. Since their work is more varied, they cannot be as expert as the individuals who work in one discipline. The complaint that the departmentalized technologist will be bored is to a great extent unfounded because in actuality she becomes expert enough to know the ramifications of her section and can then better understand and appreciate the significance and challenge of her work. This by no means belittles the necessary work of the general personnel, and an excellent general technologist is a rarer commodity than a superb specialist.

The departments of the laboratory are those of histology, serology and blood banking, chemistry, bacteriology, hematology and clinical microscopy, electrocardiography and basal metabolism, and two special ones. The divisions were influenced by the separate rooms available and are a segmentation of technologists according to separate disciplines. Certain deviations occur, however, as dictated by local custom; e.g., prothrombin times are done in the chemistry section.

In addition to the previously specifically mentioned divisions, a department concerned with special tests and laboratory research has been established. Personnel of this unit adapt special tests to specific laboratory sections performance by working on them until their sensitivity and specificity are determined, and by then a reasonable monetary value can be assigned them. Until such times the tests are performed gratis and have not been allowed to be overemphasized in the evaluation of patients' conditions. Without this department the establishment of new procedures is almost impossible at the rate these new procedures reach the literature. Even with such a department, keeping abreast of the current literature is difficult, but depending on the size of the laboratory and on the ability of its personnel, some approach to adequately developing recent tests can be made. Since some tests are described in obscure or unlikely journals, a good index of current literature is necessary. In addition to current literature the laboratory has access to much of the other major publications including books in the field. Of course, before the pathologist can discard a test as useless or impractical, he should have had it undergo a fair trial.

The use of new equipment is often commenced in the laboratory research section. With new automatic devices the routine work of the technologist may be somewhat diminished, but this only frees technologists for more complex technics. We are attempting to avoid replacing one technologist by two electronic engineers.

There is also a section of tissue culture. This came about through a series of coincidences unlikely to be repeated. Nevertheless this section can serve a multiplicity of functions. Some viral work, chromosomal studies, atherosclerosis elaboration, and tumor research can be performed here. Such a laboratory can function to a large extent as a replacement for an experimental animal unit.

The histology section is responsible for all tissues both gross and microscopic, both necroptic and surgical. Special staining technics are performed here. We have found an automatic knife sharpener and an automatic technic for preparing paraffin blocks to be very useful. The automatic technic for hematoxylin-eosin stains, although not used by our technologist, is satisfactory in my experience. This latter statement does not alter the fact that most perfectionists in the field prefer staining by hand.

The technologists at our hospital make regular rounds to collect specimens 6 times a day for the clinical laboratory sections.

The chemistry section has gone along with the modern trend for automation. An automatic device for performing certain blood chemistry determinations is available here. Without entering into the debate concerning the pros and cons of automation, suffice it to say that we have found it extremely satisfactory in accuracy and more than satisfactory in volume output. I had recommended an automatic device to the large military hospital previously mentioned, where it is considered invaluable. All blood, solid material, and nonendocrinologic urine chemical analyses are

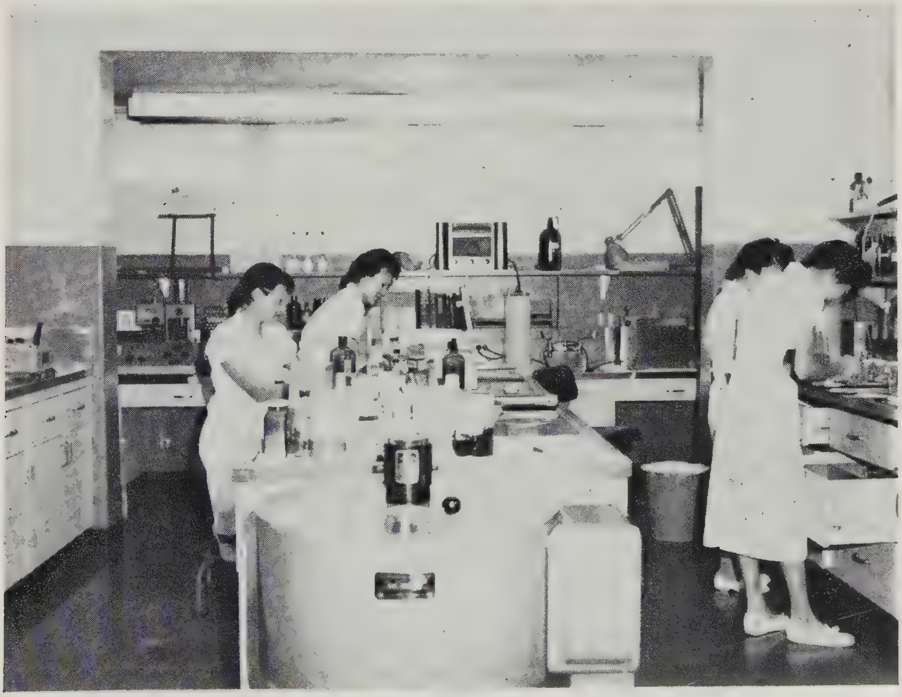

Fig. 765. Chemistry laboratory. Flame photometer is in left background. Spectrophotometer is in front and to left of flame photometer.

performed here in this section. In addition, toxicologic, electrophoretic, and chromatographic studies take place here. Wherever possible 3 standards are used along with so-called normal controls. The standards relate to low, mid, and high normal values for the material being tested. In addition, we are strong proponents of quality control. All prothrombins are standardized as regards new thromboplastin with the old batch. Precalibration technics are never utilized. Technics are micro and so-called ultramicro wherever indicated.

The section on bacteriology and mycology includes sensitivity studies. Disks are used most often and in most situations have proved adequate. Test tube sensitivity tests are performed if required. Agglutination and precipitin technics concerned with bacteriology are taken care of here. This section is extremely busy during epidemics, evaluation of new germicides and soaps, etc.

The department dealing with blood banking also deals with serology tests. We are fortunate that a statewide blood bank functions on the hospital grounds so that we have a precheck on blood type of the donor. We check the recipient's type and perform the cross match. The blood bank checks on reactions, although we also do so to a certain extent. The Coombs technics and Wiener or Grubb antiglobulin inhibition, heterophil, latex fixation, and Rh antibody determinations are done here. We perform the Kahn, Kline, VDRL, and Hinton tests. The VDRL has been a good screening test in our hands. *Treponema pallidum* immobilization and Reiter protein complement fixation tests are sent out. When we can get a good enough antigen we shall do the latter.

The department wherein hematology is performed is the one in which we do routine urine analysis and clinical microscopy. Again, we have been able to develop confidence in an automatic blood counter. This allows an accurate red cell count to be added to the physician's group of results by which he can better evaluate the patient. Since the machine actually counts the cells, the technologists are able to spend more time on differential and bone marrow counts in the knowledge that the cell enumerations will be accurate. This section also checks for ova and parasites.

Basal metabolic rates and electrocardiograms are taken care of in a special room away from the routine laboratories. Basal metabolic rates are gradually being displaced by protein-bound iodine tests, which are performed in an isolated room in the special research chemistry section. Electrocardiograms

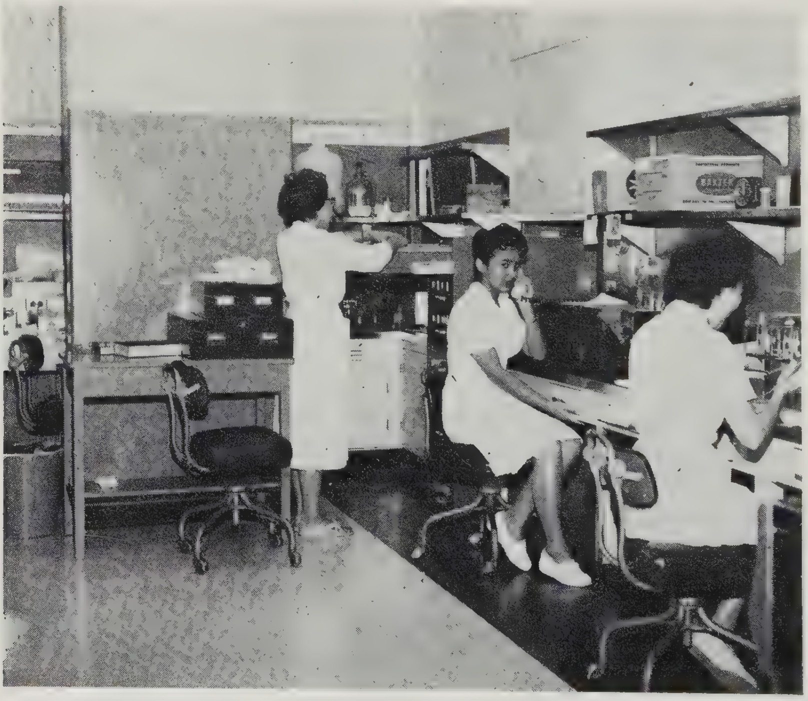

Fig. 766. Hematology laboratory. Automatic counter is on counter at right.

are often performed with a portable machine elsewhere.

All trained technologists give practical on-the-job instruction to student technologists who rotate through the various services. As the student (who spends a set amount of time in each section except special research and tissue culture) becomes more proficient, he or she is allowed more freedom in performance of more procedures. Student technologists are present only during the early shift. This is because of the lesser supervision possible by the fewer technologists on other shifts and because more detailed instruction can be given at this time by the more specialized personnel.

The pathologists select and evaluate tests and work out problems in their performance and interpretation, all in relation to the patient's clinical picture. The pathologist is a consulting physician. He should make rounds and attend clinical conferences, according to our philosophy.

This is a cursory picture of our laboratory. This review, at the request of the late Dr. Gradwohl, for whom I had great respect, will be of value if it can help laboratories either install or definitely delete any or all of the factors mentioned here according to whether they may fit any individual hospital laboratory operation.

Chapter 114

The hair in police investigation*

GENERAL CONSIDERATIONS

In an extensive paper presented before the Academy of Science of Havana, *The hair of the races in Cuba,* Dr. Israel Castellaños, Director-General of the National Bureau of Identification, Havana, Cuba, pointed out that valuable researches have been made in comparative microscopy, but there have been very few contributions concerning the hair in police technic. The most important work in this field was done in Europe. There is an abundant bibliography of anthropologic and medicolegal studies of the hair, but there are only short references to its value in police work. This may perhaps be accounted for on the basis of Gross's exclamation, in 1904: "How seldom clothes are examined, with a view to finding some hair! And if it is found, by mere chance, how seldom is it submitted to examination by an expert!"

The intensive anthropologic investigations by Castellaños on the hair of Cubans and his official position in a country in which the inhabitants have been accustomed to slaughtering domestic animals, such as pigs, sheep, goats, etc., in the courtyards, especially on church holidays and national feasts, has shown the eminent importance of hair in police science.

Gross, the Father of Criminology, has insistently called the attention of scientific investigators and of the police to the importance of hair found at the scene of a crime. "It is the duty of the magistrate," he wrote, "to give his full attention to finding and delivering such hair immediately to experts, as a hair, in spite of its insignificance, may lead us to the discovery of the delinquent." (In Austria, the magistrate exercised direction over police investigation not paralleled in English-speaking countries. Dr. Gross was a magistrate.)

Criminal investigators pay considerable heed to the points made by Gross in his very valuable book. Castellaños does not accept his doctrine with respect to examination of hair, however. Gross suggested a simple procedure for establishing the position of the tip and the root of the hair. The method is efficient with respect to that object, but it requires cleaning the surface of the hair, eliminating the dust, dirt, etc. that has settled on it.

Care in handling hair: Waldeyer, in his classic work, which appeared 20 yr. before Gross's *Criminal investigation* was published, very appropriately observed: "The dirt adhering to the hair is often more important than the hair itself." When a hair is found, therefore, it should never be rubbed between the fingers. In most cases use of a magnifying lens will enable one to determine the direction of the tip or root of the hair.

It is of utmost importance in many cases not to touch any hair found if it can possibly be avoided. Gross recommended that the hair be placed on paper, fastened thereon by means of 2 glued paper strips, and that the ends be marked with the letters A for root and B for point. At the same time a sketch is made and lettered accordingly, with letters corresponding to those of the specimen, so as to leave no doubt about the original position of the hair. In countries such as Cuba and the United States, where there is a mixed population, this may be difficult because all hair cannot be mounted in this fashion. Whereas the hair of most Caucasians can be mounted on paper in this manner, that of mulattoes will seldom permit it and that of Negroes almost never. Hairs of these people are therefore preserved in clean white papers or in small envelopes.

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*Much of the material on hair in police technic and identification through the hair by both microscopic and chemical methods is abstracted by permission from the book by Castellaños, Israel: Identification problems—criminal and civil, Brooklyn, 1939, R. V. Basuino.

Searching for hair: The smooth, lank hair of white persons and the straight, stiff hair of American Indians and Chinese are more likely to remain between the fingers of a victim than are those of other races. The more spiral the form of the hair, the less likely is it to stick between the fingers and be found in the hands of the victim, although his struggle with the aggressor may have been very fierce.

Because of the anatomic structure of its outer covering, hair sticks to clothes. The curlier it is, the tighter it sticks. This brings to mind an interesting case reported by Chief Hugh D. Harper, of Colorado Springs, Colo., at the thirteenth convention of the International Association for Identification.

> A white girl had been attacked by a Negro youth, and a suspect was arrested. The Negro people of Colorado Springs said that the accused man was innocent, the defendant's lawyer contending that the crime had been committed by a white man disguised as a Negro. The technical assistance of Dr. L. A. Bennet was requested. He found a long fair hair in the necktie of the youth, which he identified as having come from the raped girl. On the underclothing of the girl Dr. Bennet found pubic hair of a Negro, which he subsequently identified as having come from the arrested man. Thus the hypothesis of the defense was destroyed.

In America, where the population is composed of different racial types, knowledge of hair of various races is needed in police technic. The anatomic structure and the shape of the hair often indicate a definite ethnic type and thus facilitate police researches di-

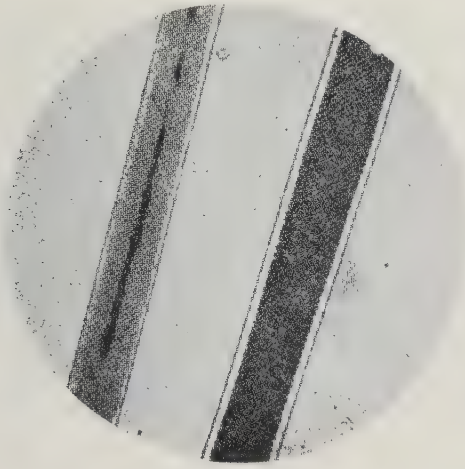

Fig. 767. Human hair (left) and animal hair (right). These are differentiated by their constituents—cuticula, cortex, and medulla. In man the cuticula is thin, slighted serrated, and imbricated. In animals it is just the opposite. In man the cortex shows small homogeneous pigment. In animals the pigment is irregular and larger than in man. In man the medullary channel is narrow, whereas in animals it is much better developed, to such a degree that the greater part of the hair consists of the medulla. (Courtesy Dr. Israel Castellaños.)

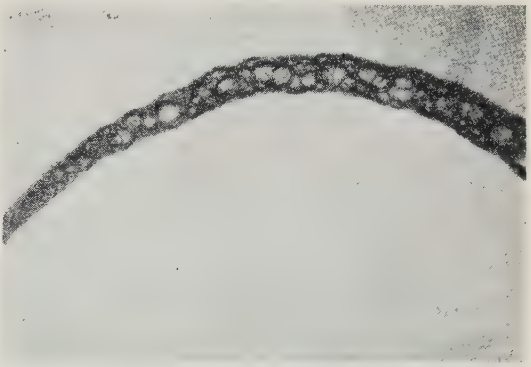

Fig. 768. Bubbles that form inside of the hair when it has been exposed to a high temperature without burning. Hair submitted to excessive heat in a permanent wave by inexpert hairdressers has an appearance similar to this. (Courtesy Dr. Israel Castellaños.)

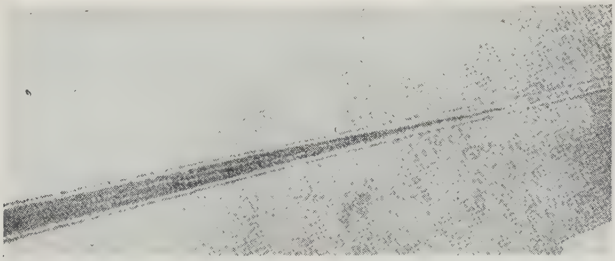

Fig. 769. The point of a hair of animal origin. Note that the structural details of its central portion do not reach the distal end or free portion of the hair. (Courtesy Dr. Israel Castellaños.)

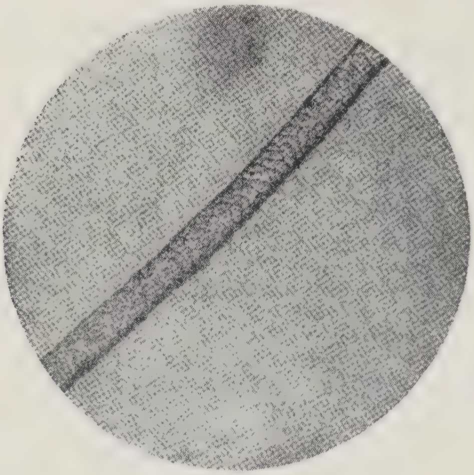

Fig. 770. The hair of a sheep. The cuticular scales are discernible in no other type of hair. Note absence of medullary channel and structural regularity of the cortex. (Courtesy Dr. Israel Castellaños.)

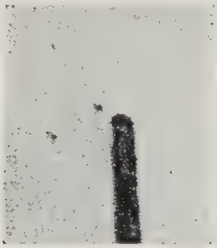

Fig. 771. Round end of a hair shaft. This form appears on hairs that have been in continual contact with clothing. (Courtesy Dr. Israel Castellaños.)

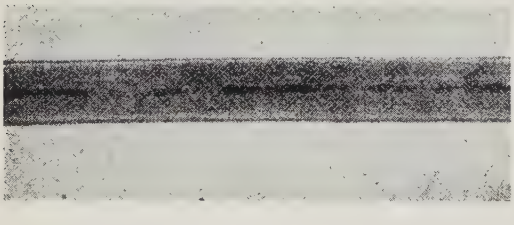

Fig. 772. A shaft in which we can clearly observe the 3 constituents of hair—cuticula, cortex, and medulla. Note lack of continuity of the medullary channel. (Courtesy Dr. Israel Castellaños.)

rected toward persons of some predetermined race. If, for example, a crime has been committed, and on the site of the crime one or more thick rigid hairs are found, it is assumed that a Chinese or an American Indian is involved. Likewise, if a kinky hair is found, some Negro must have been at the scene of the crime. Hair thus directs the investigation to persons of definite races.

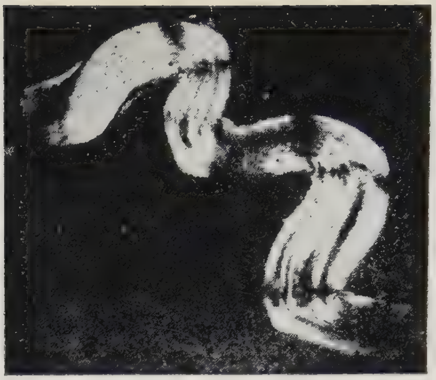

Fig. 773. Photomicrograph showing microscopic appearance of curled hair. (Courtesy Dr. Israel Castellaños.)

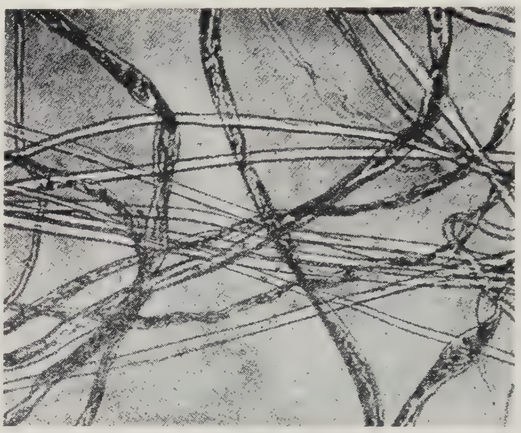

Fig. 774. Cotton and silk fibers. Note the different microscopic appearance of each. (Courtesy Dr. Israel Castellaños.)

EXAMINATION OF THE HAIR

It is advisable to keep the hair in bottles with ground glass stoppers in order not to lose its specific odor.

It is best to examine the hair as it is found. Pick it up with forceps and place it on a slide. Cover with a cover glass. Do not touch the hair with the fingers since this might remove any matter on the surface—dust, pollen, coal dust, etc.

Examine the hair under a microscope. Then, as Waldeyer recommends, without having cleaned the hair add distilled water and examine under increasing magnification. In this way one can often discover blood, spermatozoa, pus, parasites, vegetable or metallic particles, etc., all of which can be identified. There are numerous instances in which the hair is surrounded by substances which are found only on persons engaged in specialized occupations.

After having verified these preliminary observations, proceed to clean the hair with water, tissue paper, or a small brush.

Removal of pigment: If the hair contains much melanin, as is the case with Negroes and dark individuals, or if the medullary canals are full of air, it is difficult to observe the structural details of the hair or to determine the medullary index. To avoid this difficulty, authorities recommend removing the color from the hair by soaking it in nitric acid for several minutes if the examination is urgent, or in oxygenated water for several hours if there is sufficient time.

In a great many cases the action of the nitric acid is protracted, and the hair under investigation is destroyed, arguments which might be brought out by either the prosecution or the defense. J. Delgado Roig, of Sevilla, Spain, has eliminated these difficulties by using oxygenated water and nitric acid; he uses 15% sodium carbonate, in which he leaves slightly pigmented hair for 30 min.; hair with much melanin is kept 5 hr. in this solution. The hair is then mounted in Canada balsam, which is recommended by all authors. This method not only permits exact comparisons between 2 or more hairs but also identification of the hair at any later date since the bleaching reagent does not impair or destroy the hair, even though it has been mounted for considerable time.

Cross section of hair: A portion of the hair should be cut in cross section.

Cleanse the hair in 95% alcohol for 5-10 min.

Immerse in 10% sodium hydroxide 5-10 min. or longer, depending upon how soon the hair becomes softened by the solution.

Immerse in 20% alcoholic hydrochloric acid 5-10 min.

Gonzalez suggests a convenient method of sectioning hair prepared as described.

Partially incise a small segment of formalin-fixed liver on one side and stretch the hair out in the bed of the incision. The natural elasticity of the liver section will hold the hair in place. Then fix, harden, and embed the tissue in celloidin or paraffin. Cut it in the usual manner and mount the unstained section in balsam.

Gram stain of hair:

Stain with Gram stain, wash rapidly in 95% alcohol, and mount in balsam without counterstain.

The cuticula is outlined in violet wavy lines on the surface of the hair. If the hair is dark, it may be necessary to remove the pigment before staining by bleaching it with a mixture of hydrogen peroxide and ammonia water.

Improved technic for sectioning hairs—method of Kirk[1]

It is often necessary and desirable to observe the hair in cross section. This is especially true when dealing with animal hairs. The transverse aspect is often highly diagnostic of a family with modifications in the genera. The usual methods of embedding lead to an angular section, which gives a distorted picture of the true contour of the fiber. Embedding in collodion is somewhat more successful, but the collodion-coated hair must ordinarily be mounted secondarily in paraffin to make it rigid. The problem of bending and distortion is therefore a serious obstacle to properly sectioned hair.

Kirk described an improved method for sectioning hair. He advised a small microtome, developed by Hardy, with which a person after some experience can make a cross section in a very short period of time. The method combines embedding on a wood base with the use of a hand microtome which allows sections of 0.01-0.015 mm. in thickness or thicker.

Procedure:

Cut strips of balsa wood 1 cm. wide and 3 in. long from ¼ in. thick balsa planks.

On one of the centimeter-wide surfaces, apply a single layer of cellulose-acetate glue and spread out evenly to cover the wood strip completely in its central portion. Slow-drying Comet model airplane cement has been found to be readily available, convenient to use, and quite satisfactory as an embedding medium.

While the film of cement is still wet, lay the hairs to be sectioned on the cement parallel to the long axis of the balsa base.

As the film becomes tacky, apply a second layer of the embedding medium and spread so as to completely cover the hairs.

Label the block on the reverse side and place in a closed container, such as a small box, to dry slowly.

Observe 2 precautions. Do not apply more than the 2 necessary layers of cement because thicker films are much more difficult to cut; also do not accelerate the drying process since numerous and abnormally large bubbles are thereby formed which, if they surround the hair, tend to decrease the tenacity with which it is held. Upon drying, small bubbles are seen throughout the embedding medium. These do not ordinarily interfere with the quality of the final sections.

Sections may be cut within 30 min. if necessary, but they are thick and wedge-shaped since the embedding cement is not completely dry, which tends to make them yield in front of the cutting edge. At least 12 hr. or overnight is recommended as a satisfactory drying time.

Before embedding, clean and dry the hair by washing thoroughly in acetone or alcohol-ether mixture and allowing it to dry on filter paper for a short while. This process takes only a few minutes.

If a number of hairs of a known source are to be embedded on the same block, they may be oriented while drying, with all proximal or distal extremities in the same direction. This procedure facilitates handling the specimen while affixing it to the block.

When thoroughly dry, place the wood strip carrying the embedded hair in the specimen clamp of a table model hand microtome.* Adjust the microtome until the portion of the hair desired for sectioning is even with the blade holder guides. Always use a razor blade holder, designed for the instrument with a fresh sharp blade. Make the cut with a short slicing motion, through the embedding layers and hair, **but only as far as the underlying wood base.** Make the next cut in the same fashion, after advancing the block the amount desired. As many cuts may be made successively as required.

If another portion of the hair must be sectioned, adjust the block to the region of interest and repeat the cutting process. As the fiber is always in view through the cellulose-acetate layer, it is convenient to obtain sections close to the ends, in the center, or in any other area of the hair. The severed but unsectioned parts of the hair remain attached to the uncut block in their original relative position and may be recovered by dissolving away the lacquer with acetone. The block has suffered little damage and may be resurfaced with sandpaper and used repeatedly.

Using a pair of fine-tipped forceps, lift the hair sections, set in a slide of cellulose-acetate 0.5-1 mm. deep and 1 cm. wide from the block, and place in a small evaporating dish. Dissolve the lacquer with 1 or 2 ml. acetone. When the acetone evaporates, the resolidified cellulose acetate will be seen to have migrated up the sides of the dish, leaving the clean, free sections in the bottom.

Observe under a stereoscopic binocular microscope and transfer to a previously albuminized slide by touching each with the tip of a sharpened dissecting needle, to which they cling, then depositing them on the slide.

Inspect the slide briefly under the stereoscopic binocular microscope. Any sections standing on edge can be gently teased into their transverse plane with a needle.

A slightly faster procedure is to separate the lacquer slices and mount them on a slide. However, the lacquer slice surrounding the hair sections somewhat interferes with definition of the contour of the hair.

Apply a permanent mounting medium and cover slip. Clarite X dissolved in xylol is excellent. This medium infiltrates the hair and clears it very well for observation.

This technic has been useful in our hands. One potential difficulty in mounting the hair in the proper position is due to the fact that not all the lacquer is removed from the hair before mounting.[2] By using 2 or 3 washings with removal of the acetone each time, the lacquer is all removed.

POINTS IN INVESTIGATION

During the struggle that at times takes place in crimes of violence and which usually precedes the death of one or the other antagonist, often the hair or beard is grasped. Therefore, one must look for hair in the hands of the victim and on the clothes of the aggressor. If the victim died by strangulation, search not only the fingers but also the areas under the fingernails of the victim and the suspect. In cases of rape, the underclothing is examined. Most published cases refer to such findings.

A review of the cases of death by shooting shows that the literature gives little information concerning investigation of hair in these cases. Investigators often do not look for hair or examine it in cases which appear to be suicides or homicides but rely too much in certain instances on the necropsy findings. Forensic study of wounds and collection of data by the police should coincide since police technic and forensic technic complement each other.

As an example of the part played by hair investigation, Castellaños cites the following case.

> A woman, an alien, was found dead in a bathtub, the body being in a state of advanced putrefaction. There were 3 large wounds on her head. It was alleged that she had died an accidental death, having slipped and fallen while bathing, and that her skull was fractured when it struck the faucets of the tub. Public interest in the case was great; some agreed with the accident hypothesis, others insisting that this was a criminal death. Some days after the corpse had been found, Castellaños' bureau was called upon to make an investigation.
>
> A thorough search was made, and in the tub and on the walls of the bathroom at approximately the height of the corpse, police recovered an abundance of hair. This was compared with that of the victim and found to be identical.
>
> The roots were examined; they presented indisputable evidence of having been violently torn out of the scalp. Some of the shafts were bruised; others had been cut with a rather blunt instrument. The hair thus changed the situation and led the police from the hypothesis of suicide to the certainty of homicide. To complete the demonstration, Castellaños proved that there was blood in the bathtub and that this blood had flowed from the victim; there was blood on the floor of the bathroom, which had been washed. And finally, blood which the perpetrator of the crime had washed from his hands was found on the basin. When dressing before

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*Model 900, American Optical Co., Buffalo, N. Y.

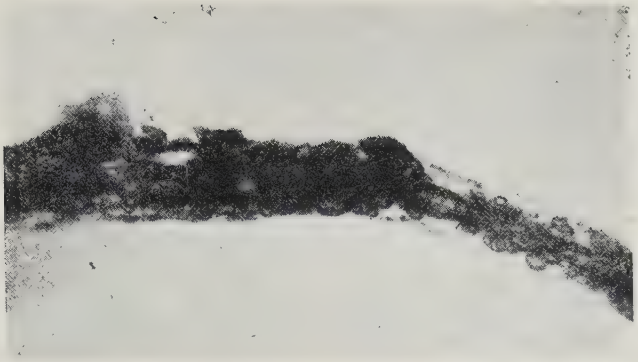

Fig. 775. Appearance of the hair from a person who committed suicide by shooting himself. The hair is burned by a point-blank shot with black powder. Note presence of bubbles produced by the heat in the mass of the medulla and the residue left by the shot on the outside of the hair. (Courtesy Dr. Israel Castellaños.)

making his escape, he had left some gray hair between the teeth of a comb, which the Cuban Bureau is still holding. The medical examiner reported that a murder had been committed, and the police confirmed this thesis.

There are a great many homicides and murders by shooting which give the appearance of suicides. On the other hand, some cases of suicide present the appearance of murder. In these cases study of the hair often helps solve such medicolegal problems.

Effect of firearm wounds on hair: The first investigator to study the effect of firearm wounds on hair was Minakow. Daake, confirming the tests of Minakow, showed that the microscopic examination of hair is decisive in determinating the distance at which the shot was fired.

If the shot is fired from a very short distance, the hair of the victim is burned. Microscopically the hair shows countless bubbles inside, but no change can be detected by the naked eye. Changes are greater after black powder has been fired than when smokeless powder is used. Schwalbe has performed magnificent researches in connection with this problem.

Temperature at which hair burns: Piedelievre and Zebouni, using electric stoves, showed that at 100° C. hair shortens and loses weight; at 150° it presents gaseous bubbles in its medulla; at 300° it is carbonized. These data are useful for determining to what temperature the body of the victim has been exposed. As an example, one case is cited.

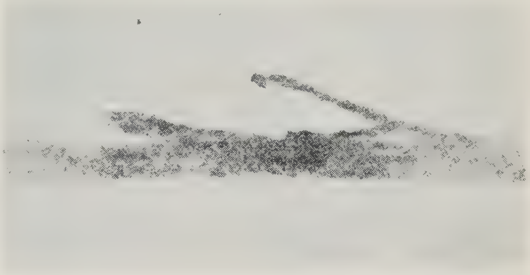

Fig. 776. In this case the shot was fired from a short distance and smokeless powder was used. The cortex has been broken and raised by the unburned grains of powder, which always escape from the barrel of pistols or revolvers. Note that the outside of the hair also shows residue from the shot. (Courtesy Dr. Israel Castellaños.)

In a village of the province of Santa Clara, Cuba, there lived a widow with her idiot daughter. The mother married a baker and moved with her daughter into the baker's shop. The stepfather forced the idiot to carry logs for the oven and in many ways was cruel to her. One day he beat her until she was almost dead. The poor idiot's cries were pitiful. After that incident she disappeared. The mother announced that she had left home, but the neighbors were inclined to doubt her story and were suspicious that the baker had murdered her. An examination of the baker's quarters was ordered. Police searched the oven, and there they found countless carbonized hairs. Upon further examination and under questioning, the baker was proved to have murdered the girl and to have tried to destroy the evidence of his crime by burning the body in the oven.

IMPORTANCE OF EXAMINING HAIR

All authors dwell at length on the different features of human and animal hair; the specific signs of hair cut with sharp instruments, of bruised hair, of hair torn out by violence, of hair that has fallen out spontaneously, in the course of its renewal; the details indicating the region or part of the body from which the

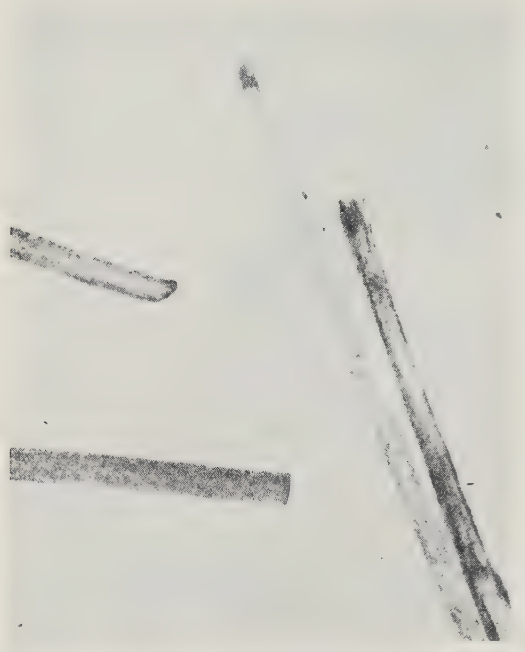

Fig. 777. The point ending in a sharp angle and the one below, perfectly flat, with clean edges, were recently cut with the sharp scissors of a hairdresser. These hairs came from a woman. The lighter points, lying vertically, which are from less pigmented hair of another woman, are split, indicating the use of tweezers. The irregular or spread ends show that it has been a long time since the hair was cut. (Courtesy Dr. Israel Castellaños.)

hair comes; the data permitting deductions as to whether it belongs to a man or to a woman; the age of the person from whom it came; and the changes due to dyeing, curling, or combing it. These are but part of the program of identification. One must remember that hair, like bloodstains, reconstructs the crime in many cases by confirming the correct and scientific findings of the autopsy, as in the case just cited. It may help to differentiate homicides from accidents; it helps to distinguish suicides and murders by proving the distance at which shots were fired; it offers definite proof in some cases of sodomy, as Kutter and Sonnenschein have shown; it may determine the race of the victim or aggressor in even the most complex and difficult cases. For these reasons a search for hair must be made in all criminal cases; it must be properly recovered and perfectly preserved so that examination of such hair will give the best possible scientific results.

Hair should be searched for not only in some types of crimes of personal violence, such as rape, strangulation, etc., where it obviously is likely to be found, but also in every crime, from robbery to murder, from infanticide to rape, from suicide to sodomy.

MICROSCOPIC EXAMINATION OF HAIR

The importance of the microscopic examination of hair in criminal investigation has been described by many authors. Rolffs in 1840 referred to the problem given the experts by a number of black hairs, obtained in 1832 in connection with a homicide. Orfila in 1835, Ollivier in 1838, Schuermeyer in 1846, Falk in 1856, Lender in 1863, Kutter in 1865, and many others thereafter have published case histories and insisted on the necessity of studying the hair in order to decide a multitude of legal cases scientifically. In the introduction to the work of Glaister it is pointed out that since 1851 British judges have considered hair to be valuable circumstantial evidence in criminal cases.

Hair is a distinctive specialized anatomic structure. It consists of a shaft, a point, and a root. That part of the hair which is not embedded in the skin is called the **shaft.** The shaft terminates in a free end called a **point.** The part that is inserted in the skin is called the **root.** The root swells into a more or less ovoid form called the **bulb.** The bulb contains an indentation, very much like the bottom of a bottle, in which is contained the **papilla,** a projection of the skin, which is the organ which produces the hair. Each of these parts—the shaft, the point, and the bulb—offers important details. A hair may be compared with a fine pin, in which the head is the bulb, the body is the shaft, and the sharp extremity is the point.

The shaft is composed of the **medulla,** the innermost core of the hair; the **cortex,** the intermediate layer which surrounds the medulla and which is the thickest and most distinctive part of the shaft; and the **cuticula,** around the cortex. The medulla looks like an irregular cylinder and contains air spaces and some scattered cells of various sizes and shapes. The medulla often disappears toward the distal end of the shaft. The very fine type of human hair might not contain a medulla. The cortex is composed of elongated, spindle-shaped, longitudinal, horny plates attached closely to each other. They contain pigment granules. The cuticula is composed of non-nucleated overlapping polygonal cells, the free edges of which are directed toward the distal end of the hair.

Age of individual from whom derived: Oesterlen gives the following average diameters of hair of different age groups:

12 days	24μ
6 mo.	37μ
18 mo.	38μ
15 yr.	53μ
Adults	70μ

Lanugo hairs on the fetus are very fine, have no medulla, and are almost colorless. The average diameter of the hair increases with age as can be seen in Oesterlen's table. The hairs of infants and children are usually finer than those of adults; they usually have no medullary cavity until after puberty and contain less pigment than do adult hairs. Gray and white hairs appear in middle life. This is due to loss of pigment from the shaft. Gray hair without pigment is usually translucent or may even be transparent. The diameter of the hair in the aged is less as a rule than that of younger adults.

It was formerly thought that the morphology of the medulla of human hair depended on the age of the individual, but E. M. Wynkoop showed that this is in proportion to the diameter of the shaft of the hair and is not due to age.

The hair of young people has a lower pigment content and a smaller diameter than that of adults. In elderly people the hair tends to become scarcer and finer, and the bulb degenerates. The shaft also shows signs of degeneration.

Race: Scalp hair is usually examined to study race. The color of the hair of the Caucasion race varies from light to dark. The shaft may be fairly straight or wavy and curly, with varying caliber throughout the length. The shaft is ovoid. The medulla is central in straight hairs or slightly eccentric in curly hair. In dark individuals of the Caucasion race the cortex contains much pigment and is dark brown. In fair individuals the hairs are lighter brown, or they may be reddish or blond.

American Indians and Chinese and other Asiatics usually have long straight coarse black hair with a deeply pigmented large shaft. Cross sections show cylindrical or triangular shafts, with the medulla centrally situated.

Hair of Negroes is usually kinky and black, the caliber varying greatly along the entire length of the hair. A large amount of pigment is to be found in the cortex. The hair in cross section is ovoid and narrow or may be almost flat. The medullary canal is eccentrically placed.

Sex: It was formerly possible in many cases to identify hair from female subjects by the length of the hair, but since women cut their hair frequently today and also shave the armpits and legs, the length and shape of the point are of much less value than formerly for differentiating hair. The best rule, as indicated by Glaister, is that a woman's hair generally shows a more delicate structure and a much finer granulation of pigment than that of a man. A study of the cross sections shows that the sexual difference in the weight of hair is due to the difference that exists in the area of cross section and the difference in the amount of pigment. M. Bernstein and S. Robertson of Chicago showed that male hair is heavier than female hair.

Region from which the hair is derived: To determine the region from which the hair is derived, consideration is given to the length, the diameter, the shape of the point, the material which covers the surface, and the shape of the cross section of the hair. Hairs from the beard are usually larger and curlier than those from the scalp. They usually show the effects of shaving, although if undisturbed will taper to a point. Hairs from eyebrows, eyelids, nose, and ears are as thick as the hair from the scalp but are much shorter and taper rapidly to a point. Medullary cavities are consistently present. Hairs from the axilla and pubic regions are fairly long and uneven, with eccentrically placed medullary canals. The hair has a tendency to curl. Hairs on the trunk and extremities vary in thickness and have less pigment than other hairs. There is a fine medullary cavity, although this may be absent.

The mere presence of certain substances indicates the region from which the hair came. A hair covered with fatty matter unquestionably came from the armpit; a curly hair, with crystals and vaginal secretion, can come only from the genital region of a female, etc.

Glaister summarized the bases of diagnosis as follows: (1) the total length of the hairs, (2) their texture and contour, (3) the character of both ends, (4) the general thickness, (5) whether sweat, vaginal secretion, seminal fluid, fecal material, food, nasal secretion, or wax adheres to them, and (6) the exact character of the cross sections.

Miscellaneous facts: Hair that has been recently cut shows the cut end more or less clean, according to the sharpness of the instrument used. At times hair is found in caps or hats, with both ends cut, one cut being recent and the other much older. In all cases when the cut is recent, the edges are clean, neat, and form more or less acute angles. After 3 days the point of the cut hair begins to become rounded or convex because of the continuous rubbing of substances against it.

Pincus estimated that an individual 20 yr. of age loses 70 hairs of his head daily; this figure decreases until the age of 50 yr. when only 22 hairs are lost each day. Hair that falls out by itself shows a full bulb, replete, as it has reached full growth. On the other hand, hairs that have the bulb hollow or excavated, because they have not attained full growth, show that they were pulled out. When the pull has been very violent, the root is found to have particles or cells of the surrounding skin clinging to it. In some cases hair that has been pulled out is found to have numerous cells of the skin adherent to the root, and the shaft is more or less noticeably bruised. This always occurs when the victim was attacked with a heavy, blunt, and crushing instrument.

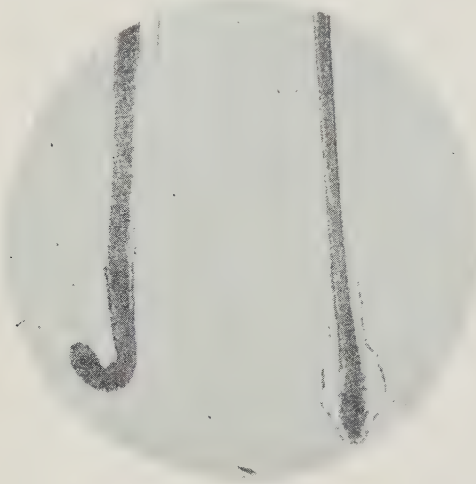

Fig. 778. Root portion of hairs that have been torn out. Note typical appearance of the bulb. The one at the right carried part of the follicle sheath. (Courtesy Dr. Israel Castellaños.)

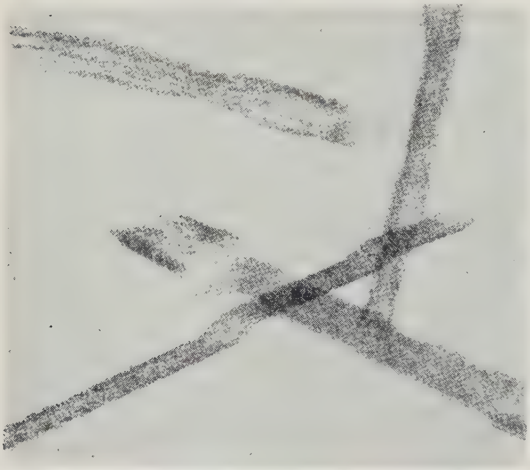

Fig. 779. Microscopic appearance of several hairs that have been struck with an instrument or weapon without a cutting edge. It can be seen how part of the hair shaft is bruised. (Courtesy Dr. Israel Castellaños.)

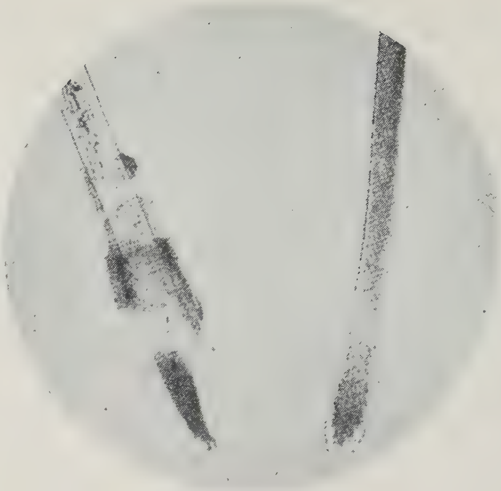

Fig. 780. Root portions of 2 hairs. The one at left was torn out and the one on the right fell spontaneously. Note typical appearance of the terminal portion in this fallen hair. (Courtesy Dr. Israel Castellaños.)

Bleached or dyed hair: Bleaching of the hair is more difficult to determine than the fact that the hair has been dyed. Dyes are easily discovered, because they leave a homogeneous or irregular layer on the periphery of the hair, which can be seen perfectly under the microsrope. Tests for determining the dye used are based more on chemical analysis than on microscopic examination.

Dyes of a metallic salt base, bismuth, silver, or lead, or those having a base of organic substances, such as vegetable dyes, can be determined by chemical analysis.

Many criminals do not hesitate to make use of surgical means to disfigure themselves and thus evade the action of the police; they may also use wigs for that purpose. It is necessary, therefore, to be able to distinguish between hair that has recently fallen out and false hair.

PROCEDURE FOR EXAMINATION OF HAIR

If one finds hairs in the hands of the victim which the victim pulled from the head or beard of his aggressor during a struggle, note must be made of the position of the hairs in the victim's hand. For greater exactitude, as Gross suggested, make a drawing of the hand to fix that position exactly. It is often possible to tell the root at a glance by the enlargement of the bulb, which is always visible with an ordinary magnifying glass. It is then unnecessary to take steps to decide where the point lies and where the root is.

The material adhering to the surface of the hair should not be dislodged since, as Waldeyer stated, "often the dirt adhering to the hair is more important than the hair itself." For this reason, any hair that may be found must be neither rubbed nor cleaned.

The hair from the victim should be placed in bottles labeled Victim, and any hair from a suspected aggressor should be placed in bottles labeled Suspect.

When no hair is found in the hands of a dead body and none is found at the scene of the crime or in the place where the victim was found, search should be made of articles of personal use, such as towels, combs, etc. Hair commonly adheres to clothing, not only to caps and hats but also to stockings. It can frequently be found in the nap of towels, and it may be in the hair of the victim or of the aggressor. It is rare not to find hair in the teeth of combs. Such hair may be from the deceased when he last used the comb, or it may have come from the aggressor if he combed his hair before leaving the scene.

Hair may also be found on sheets and pillow cases. It consists of hair that has fallen out while the victim was resting or sleeping in the bed or hair that has been pulled out violently in the struggle which preceded death.

Hair comes out not only because of the periodic renewal to which it is subject but also because of a tug given by an individual in defending himself or by the aggressor in an attack. The hair falls unnoticed and remains at the scene of the crime as a silent witness to the tragedy and as circumstantial evidence of the crime. If the investigator searches for it with a magnifying glass, he will find that it contains a varied number of substances, according to the nature of the crime: blood from a mortal wound, black or smokeless powder from a fatal shot, semen, etc.

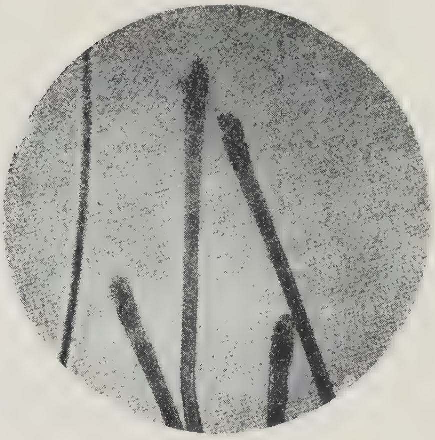

Fig. 781. These hairs show that in fallen hairs the root sheath is missing. In cases of falling hair, part of this sheath always remains embedded. (Courtesy Dr. Israel Castellaños.)

Finding a hair, according to Glaister, makes it necessary to answer these questions.

1. Is the hair that of a human being?
2. If it is human hair, is it the hair of a male or a female subject?
3. What is the approximate age of the individual from whom the hair has been derived?
4. From what part of the body has the hair been derived?
5. Has the hair been dyed or is it of natural color?
6. Has the hair fallen out, been pulled out, or cut?
7. If it has been cut, was a sharp or a blunt instrument used?

Lambert and Balthazard suggest the following program.

1. Is the hair that of a human being or of an animal?
2. From what part of the body does the hair come?
3. Is the hair from a male or female individual?
4. What is the age of the individual from whom the hair came?
5. Was the hair cut and at what time?
6. Was the hair pulled out, or did it fall out accidentally?
7. Is the hair dyed, and if so, by what process?
8. Did the hair under examination come from a living person?
9. What is the identification of the hair?

Castellaños' questionnaire is longer because he makes additional examinations to determine the following facts.

1. The trade of the individual from whom it came.
2. The distance at which the fatal shot was fired in cases of gunshot deaths.
3. The kind of powder used in the fatal shot in such cases.
4. The poisoning of the subject from which it came.

HUMAN AND ANIMAL HAIR

Most authors begin the investigation by determining whether the hair found is from a human subject or from a lower animal. At present there is no one known identifying specific characteristic of human hair, just as there is no illness which can be diagnosed by a single symptom; but the hair of no one animal combines the various anatomic characteristics found in the hair of man. In the outline that follows the characteristics of human and animal hair are compared (see also Figs. 767, 769, 770, and 782).

- I. Medullary (center) channel
  - A. Man
    - 1. Aerial net with fine granules
    - 2. Invisible
    - 3. Medullary cells not previously dissociated
    - 4. Value of medullary index (relation of diameter of the medulla to shaft diameter) inferior to 0′ 30
    - 5. Downy hair without medulla
  - B. Animal
    - 1. Aerial content of vesicles more or less voluminous
    - 2. Medullary cells very apparent
    - 3. Value of medullary index over 0′ 50
    - 4. Medulla in steps in the downy hair
- II. Cortical (outer) substance
  - A. Man
    - 1. Constitutes a thick muff
    - 2. Pigment in very small homogeneous granulations
  - B. Animal
    - 1. Constitutes a very thin tube
    - 2. Pigment in irregular granulations; always larger than those of man
- III. Cuticula
  - A. Man
    - 1. Thin scales; lightly serrated (sawtoothed); strongly imbricated (tilelike)
  - B. Animal
    - 1. Thick scales; less imbricated than in man; markedly serrated

INDIVIDUAL IDENTIFICATION OF HAIR

It is after having settled all the points just discussed that the investigator undertakes what may be properly called the identification of the hair, that is to say, the individualization thereof, or, in other words, the determination of whether the hair found comes from a definite suspect or from a given individual.

Measurements are taken of the length of the hairs under comparison and their average diameter, and the maximum measurements are noted. If the different data coincide and the pigmentation of the hair is identical, as determined by the Fischer chromatic scale, one has reason to believe that the origin is the same. To make the examination definitive, minutely study the individual signs or characteristics of the hair. Observe the peculiarities of the **medullary canal** and the **point.** The coincidence of wear or degree of rubbing of the free point of the hair, with the coincidence of the length and the coincidence of the diameter, consolidates the identifying diagnosis. The coincidence of arrangement, abundance, and coloring of the pigmentary granulations makes identification or individualization of the hair certain.

In addition to these details, purely anatomic, there are many others that contribute to make identification of the hair more eloquent, such as racial characteristics, the existence of identical vegetable substances, metal particles, pathologic changes, parasites, etc.

The presence of foreign particles, such as coal, pollen, etc., may prove to be indications of the trade in which the individual was engaged.

Finally, it has been shown that with adequate chemical methods, presence of arsenic in the hair can be shown when the individual has been poisoned with arsenicals.

It may be concluded that microscopic, chemical, and bacteriologic study of hair offers a fertile field of criminal investigation. From Castellaños' experience of many years he has found on numerous occasions that

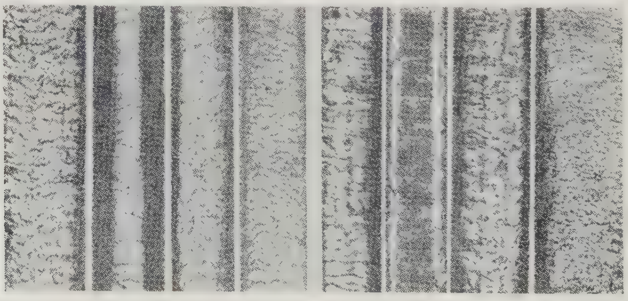

Fig. 782. Hair characteristics. Left to right, hair of Negro, Mulatto, white man, white woman, dog, cat, horse, and pig. (Courtesy Dr. Israel Castellaños.)

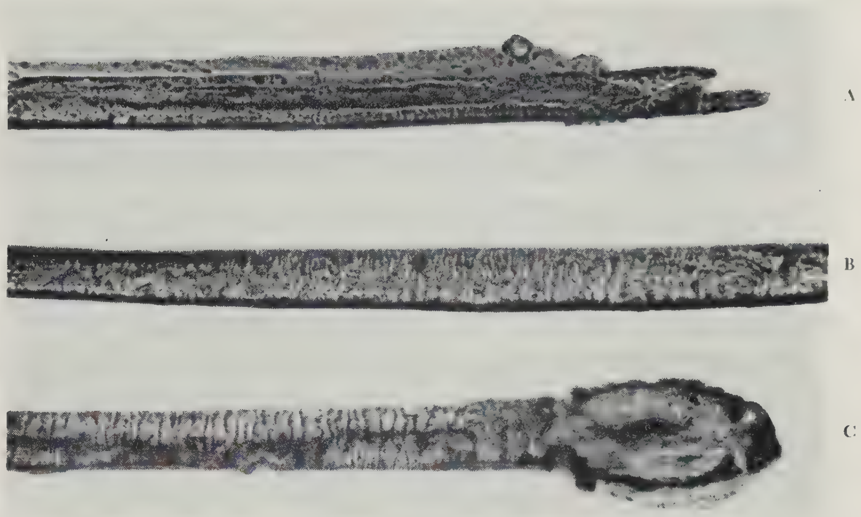

Fig. 783. Exhibit A, Case 1. **A,** Photomicrograph of the severed end of the hair found in the slit of a screen door. **B,** Photomicrograph of the middle portion of the same hair. **C,** Photomicrograph of the root of the same hair. (Courtesy Dr. Israel Castellaños.)

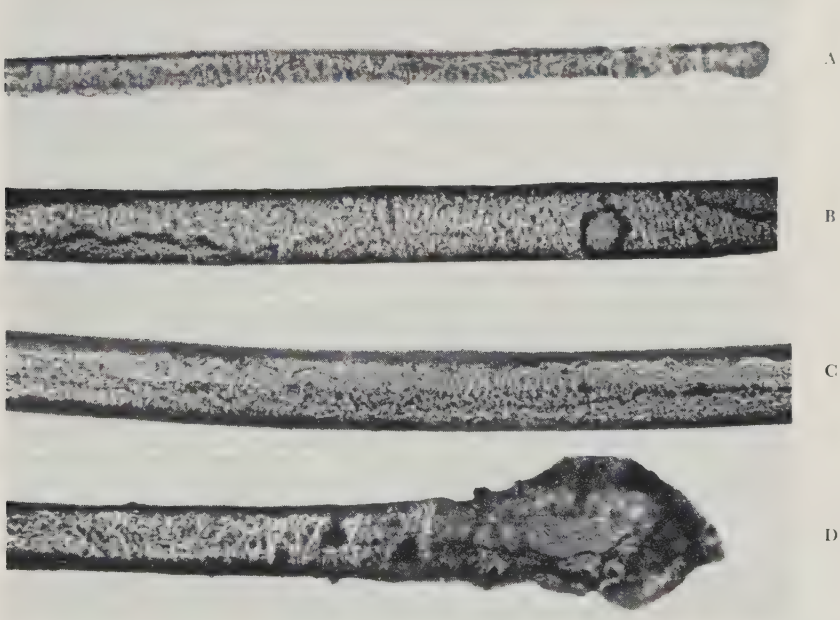

Fig. 784. Photomicrographs of one of the hairs taken from the left arm of suspect B, Case 1. **A,** End portion of the hair. **B,** Portion near the middle of the same hair. **C,** Portion beyond the middle of the same hair. **D,** Root of the same hair. (Courtesy Dr. Israel Castellaños.)

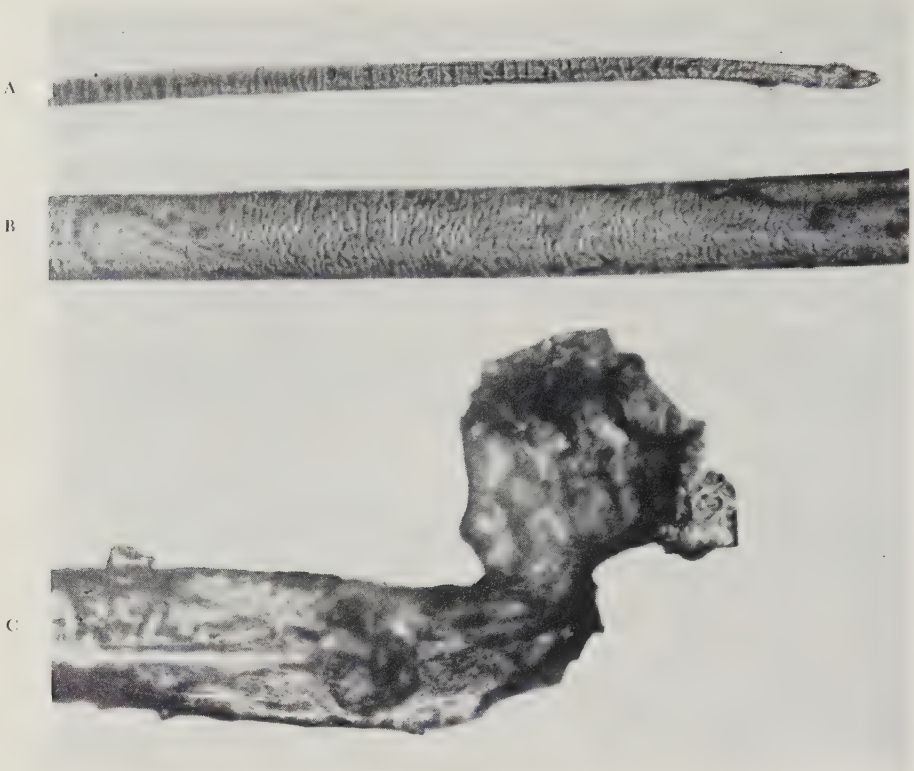

Fig. 785. Photomicrographs of one of the hairs taken from the left arm of suspect C, Case 1. **A**, End of the hair. **B**, Middle portion of the same hair. **C**, Root of the same hair. (Courtesy Dr. Israel Castellaños.)

when the usual investigation had failed and the criminal seemed about to be freed, he was able, from a few hairs which he had obtained in some cases weeks after the tragedy, to identify the persons involved in the crime and to deliver the guilty to justice.

In a personal communication, May, 1947, Dr. Castellaños furnished R. B. H. Gradwohl with records taken from his own files of cases in which the identification of hair contributed an important part to a medicolegal procedure.

Case 1: In Louisiana, the screen door of a camp hut was found broken. Investigators found a hair caught in the broken screen wires and, using the necessary precautions, took it for examination. Specimens of hair were also taken from suspected persons, those near the camp hut during the night. These were submitted for comparison.

The hair taken from the slit in the screen door was labeled Exhibit A (Fig. 783). Those from the 2 suspects were labeled, respectively, Exhibit B and Exhibit C. Examination of Exhibit A established the following:

1. That said hair is human hair.
2. That it came, not from the head, but from an extremity.
3. The color of the hair is light blond.
4. The free end of this hair appeared to have been cut transversely by an edge which was rather thick and not extremely sharp. If the hair had been cut by a very sharp edge the free end would show a straight, clean cut instead of the jagged cut, as seen in Fig. 783, *A*.
5. Examination of the full length of this hair revealed that the stem was not injured or compressed, since all of its elements were intact. The regularity of the hair and the integrity of the cuticula can be seen in Fig. 783, *B*. The diameter varied from 22-56μ.
6. That the root of this hair presented all of the characteristics found in hairs which have been pulled out, as is seen in Fig. 783, *C*.

Examination of Exhibits B and C showed the following:

1. That these are also human hairs taken from the extremities.
2. That Exhibit B comprised 9 hairs the length of which ranged from 8-28 mm.
3. That Exhibit C comprised 5 hairs the length of which ranged from 11-27 mm.
4. That the diameter of the hairs in Exhibit B varied from 14-66μ.
5. That the diameter of the hairs of Exhibit C varied from 12-134μ.
6. That the hairs taken from Exhibit B were light like those of Exhibit A, whereas the hairs taken from Exhibit C were darker or pigmented.
7. That the similarity of color of the hairs of Exhibits A and B was clearly visible, and micro-

scopic comparison proved that the tone or chromatism of both exhibits was identical.

8. That although the disparity between Exhibits A and C was clearly visible to the naked eye, microscopic comparison proved the chromatic discordance between these exhibits.

9. That through macroscopic and microscopic examination and the micrometric measurements taken of the 9 hairs sent as Exhibit B, their anatomic structure, characteristics of cuticula or outer covering, the nature of the medullary structure, and the tone and arrangement of pigmentation proved that they were identical to those of Exhibit A.

10. That through all of the aforementioned anatomic details it was proved that the 5 hairs sent as Exhibit C were completely different from those of Exhibit A.

11. That all the scientific observations made proved that all the macroscopic, microscopic, anatomic, metric, and chromatic characteristics found in Exhibit B are also found in Exhibit A, whereas the contrary is true of Exhibit C.

12. That all these data coincided to establish the fact that the hairs taken from the left arm of Suspect 1 were exactly the same as those found in the slit in a screen door in the rear entrance to a camp hut. Fig. 786 shows that the hair of Exhibit A coincides exactly with that of Exhibit B as registered by examination with the Spencer comparison microscope.

Case 2: A man was struck and killed by a motor vehicle. A passenger bus had been seen in the vicinity at about the time the man was killed. The driver of this bus was questioned by the police. He stated, under questioning, that he had felt a slight jar at about the place where the body had been found. It was quite dark. He had stopped his bus and looked around and, finding nothing wrong, had driven on.

An examination of the bottom of the bus revealed the presence of one strand of hair and a small fragment of what appeared to be human tissue. Later a quantity of hair was taken from the dead man's head for comparison.

Castellaños received 2 specimens marked, re-

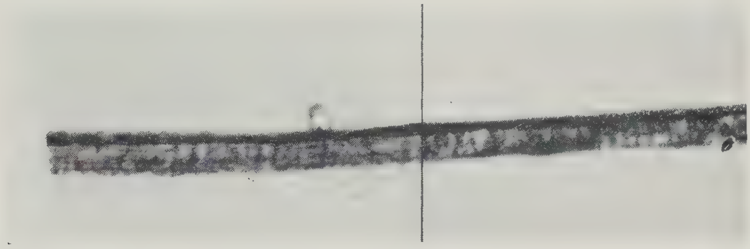

Fig. 786. Photomicrographs of Exhibit "A" and "B," Case 1, as seen under the Spencer comparison microscope. (Courtesy Dr. Israel Castellaños.)

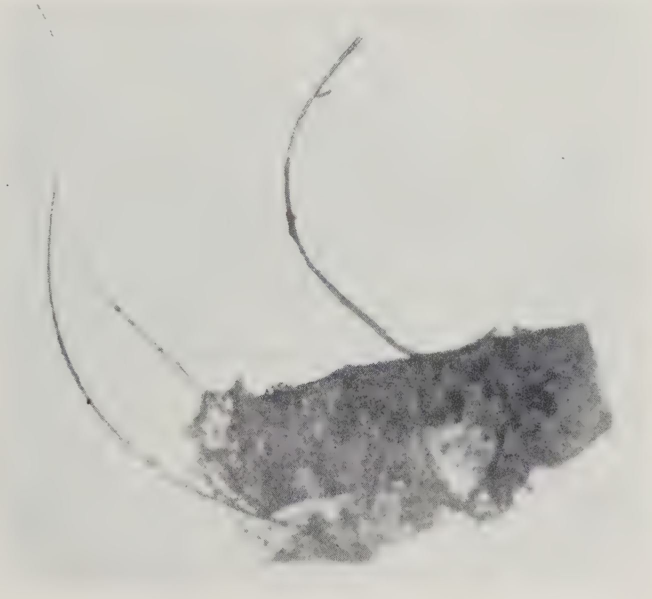

Fig. 787. Tiny fragment contained in envelope marked "Questioned." Case 2. (Courtesy Dr. Israel Castellaños.)

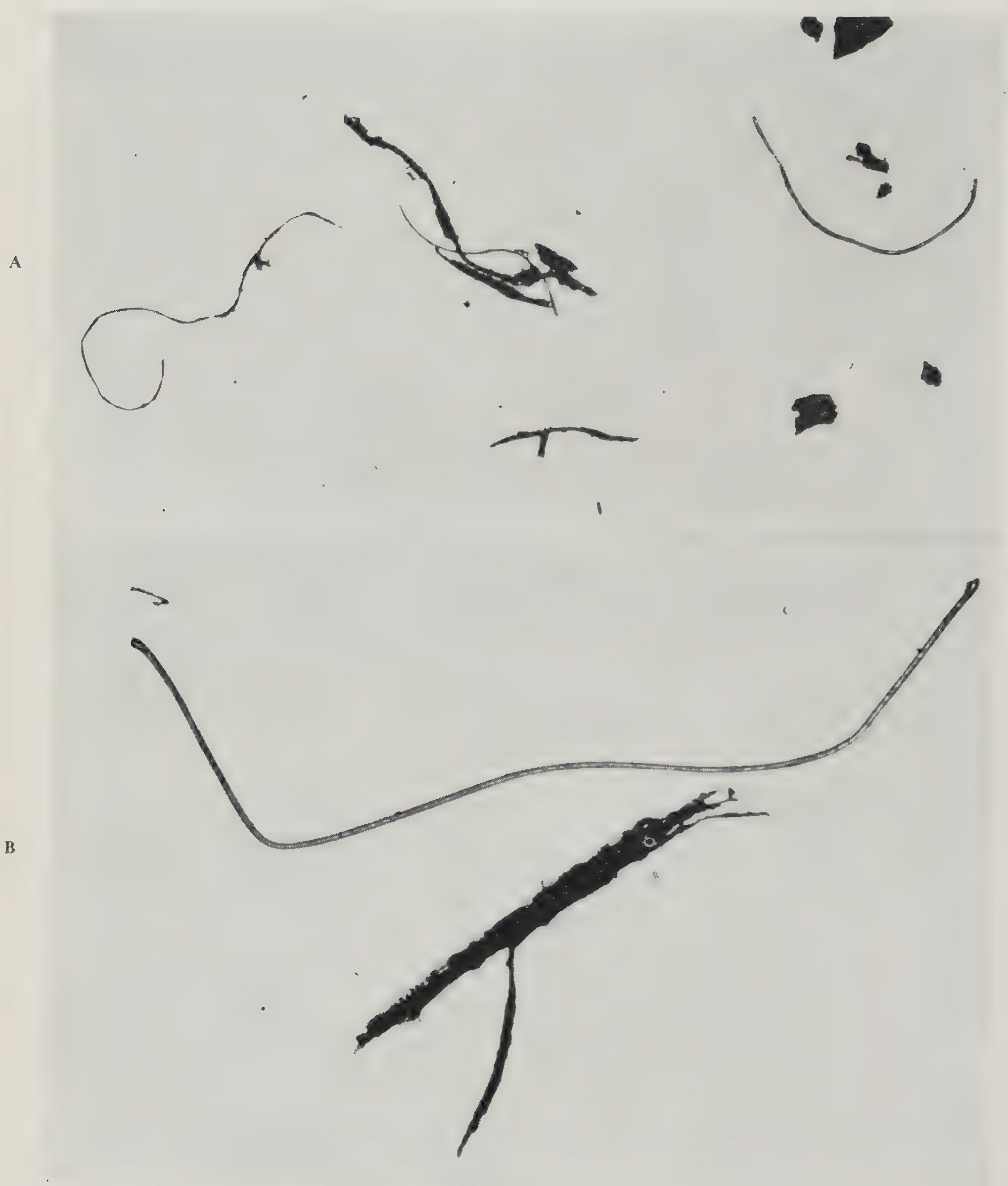

Fig. 788. Contents of envelope marked "Questioned," same as Fig. 787. (Courtesy Dr. Israel Castellaños.)

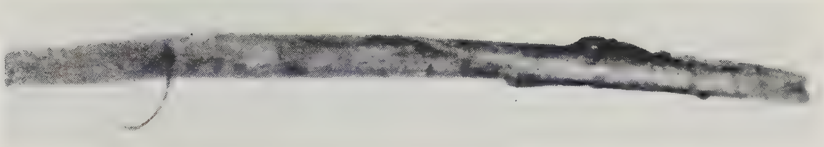

Fig. 789. Enlargement of fragment (Fig. 788), which reveals it as connective tissue. (Courtesy Dr. Israel Castellaños.)

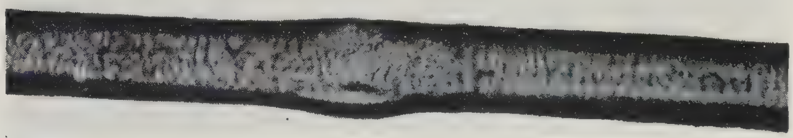

Fig. 790. Body of hair showing crushed portion on the right-hand side. Case 2. (Courtesy Dr. Israel Castellaños.)

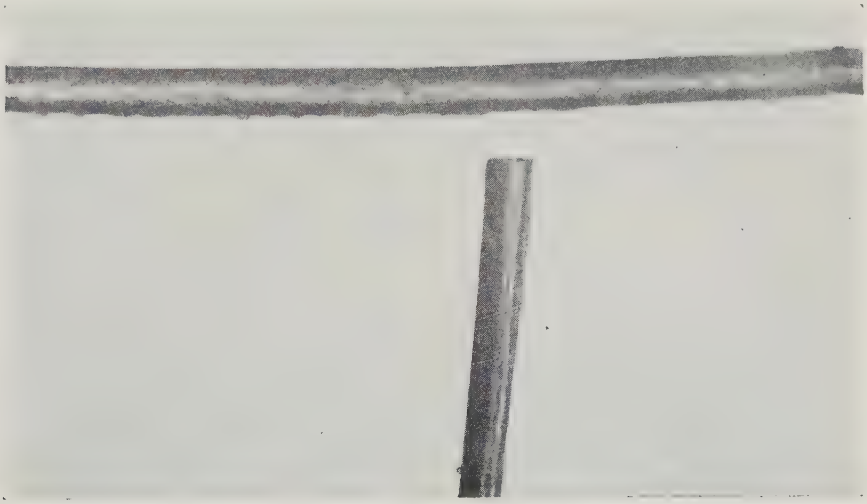

Fig. 791. Portions of fragments revealing normal hair structure. Case 2. (Courtesy Dr. Israel Castellaños.)

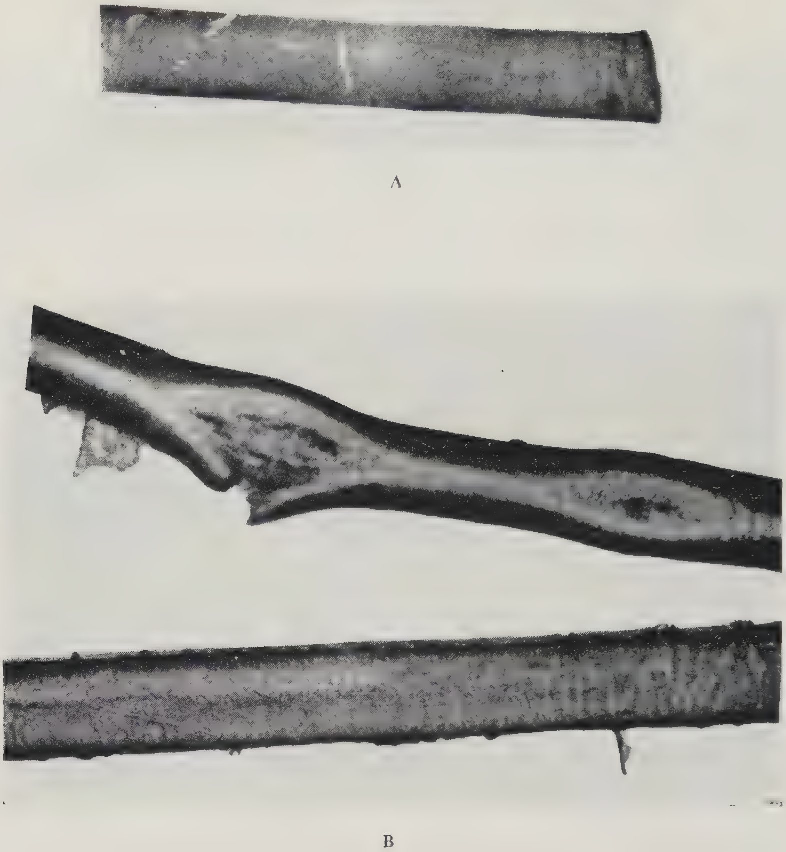

Fig. 792. A, Hair showing torn cuticula. **B,** Hair showing greenstick fracture of the stem. Case 2. (Courtesy Dr. Israel Castellaños.)

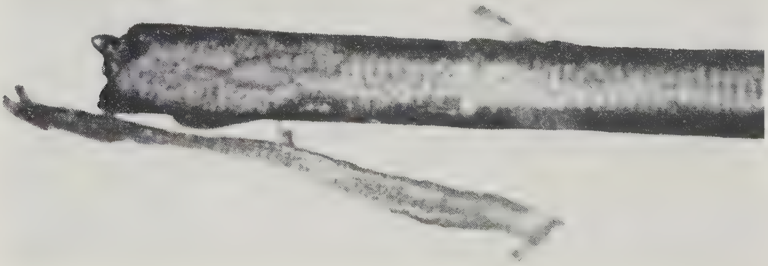

Fig. 793. Hair that has been crushed and broken, showing mechanical action. (Courtesy Dr. Israel Castellaños.)

spectively, Victim and Questioned. The envelope marked Questioned contained the tiny fragment seen in Fig. 787. This tiny fragment, when placed upon slides, showed 2 objects, the details of which may be noted in Fig. 788.

The dirt was removed from the small organic fragment by washing it in saline solution which does not alter the anatomic structure or the color. The cleansed fragment was then prepared for microscopic examination, which revealed that the fragment was connective tissue, as seen in Fig. 789. The preliminary microscopic examination revealed that the material clinging to the small fragment was hair and not fiber.

As can be seen in Fig. 788 the hairs which were clinging to the fragment were dirty. Before they could be submitted to complete anatomic examination, they were thoroughly cleansed in saline solutions. Saline solutions make not the slightest alteration in the structure or coloring of hair. These hairs, removed from the connective tissue, were 1-2 cm. long and were arched. The hairs terminated sharply or in a point. The stem or body of the hairs was bruised, crushed, or broken. Fig. 790 shows a crushed portion on the right-hand side. There are portions or fragments, however, in which the structure is normal, as seen in Figs. 791 and 792.

The hairs removed from the small piece of connective tissue were examined under natural and transmitted light and classified according to the Bertillon chromatic classification as light brown.

The cuticula of these hairs, especially in the stem or body, was definitely imbricated. The end, which is the portion opposite to the root, was disintegrated; parts were lacking so that the cortex was exposed. Such disintegration shows that these hairs had been altered mechanically by repeated friction and crushing. The stem of one of the hairs showed a greenstick fracture, in which the body of the hair is split open. The medulla was disunited in some regions and in others it was not visible but showed a granular airlike network.

The examination of the diameters of the shortest hair, approximately 1 cm. long, showed the following: maximum diameter, 98μ; minimum diameter, 17μ; average diameter, 56.1μ; medullary index, 0.25.

Measurements of diameters of the longest hair, approximately 2 cm. long, showed the following: maximum diameter, 98μ; average diameter, 56.1μ; medullary index, 0.25.

The hairs of the Victim were light brown according to the Bertillon chromatic scale. Since all of these hairs had been cut, none of them showed the root. These hairs had a definitely imbricated cuticula. On some it was torn, as seen in Fig. 792, *A*, but there were large portions in which the hair was perfectly normal.

As seen in Figs. 792, *B*, and 793, some of the hairs were crushed and broken, thus showing enormous mechanical action. There are large portions, however, in which normal anatomic structure can be observed.

Diameters of the hair coming from the Victim were as follows: maximum, 73μ; minimum, 17μ; average diameter, 41.5μ; medullary index 0.23.

The medulla of these hairs was disunited in some regions and invisible in others.

In comparing the results of the examinations of hairs marked Questioned and Victim, there can be noted a marked resemblance in the type of the cuticula cells (definitely imbricated) and the pigmentation or coloring. Furthermore, the lesions in the hairs of the Questioned are similar to those in the hairs from the Victim.

Diameters are not the same in the 2 specimens, but such discrepancies are found when the hairs come from different regions of the body. If hairs from different regions are compared, measurements are of secondary value. Anatomic characteristics, color, pigmentation, the morphologic type of hair, etc., are the decisive factors in making the diagnosis. In Case 2 all details are alike except the measurements.

The fragment of connective tissue was too small to be submitted to all the biologic tests. There was not enough to test it serologically to determine whether it was of human or animal origin. It was found with human hairs clinging strongly to it, and the histologic examination does not deny that it is of human origin so that it can be assumed to be human.

Investigations of hairs are always slow, but such investigations are extremely important.

Chapter 115

Solutions for the preservation of museum specimens

Kaiserling method

The Kaiserling method gives by far the best results for the ordinary pathologic and anatomic preparation when the color of the red blood cells is to be preserved. If any of the faint yellows or greens (due to bile staining or other causes) are to be kept, one of the chloral hydrate fluids, preferably Klotz, gives excellent results, with Kaiserling solution III as a permanent preservative.

Specimens must be obtained fresh from the autopsy or operation and must not be allowed to soak in water for more than a few moments. Only the superfluous blood should be washed away, remembering that the one color available for preservation lies in the hemoglobin of the red blood cell, and that this, therefore, must be preserved. The basis of the methods of color preservation which are to be recommended lies in the chemical change produced by the action of formaldehyde upon the hemoglobin of the red cell and its subsequent fixation as the red alkaline hematin by alcohol.

It is necessary to enlarge on the proper laying out of the specimens in the solution I. No cloth, metal pins, or paper must be allowed to come in contact with them. Cotton wool to hold in shape and stretching on glass frames with cotton thread is all that is necessary. Large bodies of fresh fluids must be used. After careful comparison both abroad and at home, the following process has been found to give the best results.

Kaiserling solution I:

Potassium acetate	170 gm.
Potassium nitrate	90 gm.
Formalin (water white formaldehyde), 40% . .	1600 ml.
Water	8000 ml.

Care should be taken to use only high-grade chemicals.

1. Water white formaldehyde must be as free from formic acid as possible. It is not necessary to buy the chemically pure grade. A white deposit, due to freezing (paraformaldehyde) is sometimes seen in the formalin. This lessens the concentration of formaldehyde gas, and consequently the color and fixing processes are not correct. Always buy formalin in the spring of the year and see that there is no white deposit present. Do not boil it unless the amount of formalin used in making the solution is restored.

If the formaldehyde is found to be acid, it can be neutralized with sodium carbonate or sodium hydroxide.

To make a 2% (w/v) formaldehyde solution:

Formalin	5 parts
Water	95 parts

4%: Use 10 parts formalin to 90 parts water.

6%: Use 15 parts formalin to 85 parts water.

2. Potassium acetate. We recommend Mallinckrodt potassium acetate.
3. Potassium nitrate.
4. Glycerin, water white, c.p. Do not use dynamite glycerin since it produces a change in color.
5. Chloral hydrate.

The length of time the specimen should remain in the Kaiserling's first fixation fluid has caused a great deal of comment. It is of secondary importance to thorough hardening. The specimen should be fixed so that no blood will ooze out when firmly pressed between the fingers. In fact, good color results have been obtained in specimens that have stood 2-4 mo. in the solution I. These are, of course, exceptional cases. Intestines should not be left for more than 10-12 hours. Other specimens or organs should be left in the solution 1-5 days. In all instances they must be thoroughly fixed. Formalin increases the firmness of tissue. The tissue loses its natural color and assumes an aged and grayish appearance.

Great care must be taken to wash the specimens thoroughly in running water after removal from this solution.

Kaiserling solution II: Use 95% alcohol. It may be used at once without the intermediate stage of 80% alcohol recommended by Kaiserling. The specimens must be left here until the natural colors, which have disappeared under the action of the formalin in the first solution, return, the process being watched in the same way as in the development of a photographic plate.

The strength of the alcohol should not be allowed to go below 80%. To avoid this the specimens should be transferred to a second alcohol of the original strength. The time in which the specimens remain in the alcohol varies according to the particular character of the tissue.

In this stage again the specimens must be carefully washed in water; no traces of the formalin or alcohol used in the first and second solutions are to be carried to the final fluid. If either the alcohol or the Kaiserling III solution used gives off any odor of either formalin or alcohol, it must be thrown away. This is absolutely necessary.

When specimens have been left in alcohol for too long a time, color can be restored by leaving them in equal parts of Kaiserling solution III and glycerin for 24 hr., allowing for the amount of glycerin already in the Kaiserling fluid.

Kaiserling solution III:

Potassium acetate . .	4 lb.
Glycerin	2000-4000 ml.
Distilled water . . .	10,000 ml.
Phenol (preservative)	20 gm.

Kaiserling solution I is not satisfactory for preserving veterinary or anatomy specimens. The best results have been obtained by Klotz, increasing the formalin possibly to 6%, then placing in the alcohol, and preserving in Kaiserling solution III.

When specimens in Kaiserling solution III in open jars are used to pass around a class, care must be taken not to allow the surface to dry or the fluid to be contaminated with molds. One infected jar may cause contaminations in many others and necessitate putting them through solutions I and II just as would be done with a fresh specimen.

To clarify Kaiserling solutions: To clarify Kaiserling solution III, connect a 5 gal. vessel with an air compressor. Run the Kaiserling solution through a Berkefeld filter. This takes 20-30 min. Run water through the filter to clean it. Be sure to connect the filter with the water main.

Another method of clarifying Kaiserling solution III is as follows: For 10,000 ml. Kaiserling solution III, 5 funnels are required. Prepare filter paper. Do not fold it. Place the 5 filters in a row. Use 1 tbs. charcoal (very fine bone charcoal) for each funnelful. Put the charcoal in a jar and shake well with Kaiserling solution III. Run the solution through the funnels slowly. Refilter the first filtrate that comes through the funnels.

In making Kaiserling solution III do not use tap water. Use distilled water; tap water contaminates the solution.

Kaiserling solution III dissolves plaster of Paris.

Klotz formerly used a little formaldehyde in Kaiserling solution III. This is not recommended as it causes an undesirable chemical reaction. Phenol is now used. It makes a specimen slightly gray but prevents the growth of fungi.

Important points to remember: There must be no odor of formaldehyde or alcohol in Kaiserling solution III. Wash out both of these materials thoroughly so that no trace of odor remains.

Overharden in a Kaiserling solution I rather than underharden.

Always sacrifice color to permanency.

Avoid infecting the jars. If a jar is infected, the specimen must be treated again with Kaiserling solutions I, II, and III.

Formalin will not penetrate either liver or lungs. In order to fix and preserve color, these specimens must be either cut into slabs or injected.

For red blood cell color preservation, place the specimen in a Novy jar and cover with Kaiserling solution I. Then saturate with carbon monoxide gas from any gas jet. To eliminate excess gas, burn off the exhaust or connect a tube to the Novy jar and extend it out of the window.

Always protect a specimen from the direct rays of the sun even after it is completed. Colors are affected by sunlight. Diffuse sunlight will not affect them. Heat will destroy color. Keep specimens in a cool place.

Never preserve a specimen in pure glycerin. A spleen preserved in pure glycerin will shrink to ½ its size.

Be careful of the concentration of alcohol in Kaiserling solution II because it will lose its strength. Keep above 80%. Use a small alcoholometer employing a small test tube.*

Frost sugar solution

Frost sugar solution is sometimes used as a preservative.

Sodium fluoride	80 gm.
Chloral hydrate	80 gm.
Potassium acetate	160 gm.
Cane sugar	3500 gm.
Saturated thymol water . .	8000 ml.

Sodium fluoride many be omitted. Never under any circumstances use beet sugar.

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*Arthur H. Thomas Co., Philadelphia, Pa.

Chapter 116

Semen appraisal

The examination of seminal fluid with special regard to defective spermatogenesis in otherwise healthy men has been elaborately studied by Cary and Hotchkiss.[3] They present a method of fixing and staining a microscopic specimen which differentiates component parts of the cell without distorting the protoplasm and enables any physician familiar with the use of the oil immersion objective to recognize and classify abnormal sperm cells and in specimens of doubtful character to count the percentage of these cells as an added index of deficiency or improvement.

The examination of the semen is divided into 3 steps: (1) collection of the specimen, (2) gross or macroscopic investigation, and (3) microscopic study.

GENERAL CONSIDERATIONS

Collection of specimen

Collection of the specimen may be accomplished by any one of the following 3 methods, named in the order of preference: (1) specimen produced at the office of the examiner, (2) specimen collected outside the office of the examiner by external emission, and (3) specimen collected by the condom method.

Specimen produced at office of examiner: If accuracy is stressed, and a quiet, suitable room is provided, many men are not averse to this method, and the physician is in constant control of the ejaculate.

Collection outside office of examiner by external emission: For this purpose the patient is given a glass graduate and a small (1 oz.) wide-mouthed bottle. After collection in the glass graduate, the specimen is allowed to liquefy so that it will not adhere to the glass surface, and it is transferred to the bottle, which is then corked, placed under the clothing about the torso, and brought to the office.

Condom method: Patients are advised to prepare the condom in advance by washing the preservative powder from the surface that is to receive the specimen and carefully drying it. This precaution is taken because not only do foreign bodies hinder the microscopic examination, but there is also a chance of harm to the specimen by the various ingredients in these powders. After coitus the condom is tied, gently wrapped in a handkerchief (not clinging, absorbent cotton), concealed under the garment next to the skin, and thus delivered.

Patients are warned not to attempt artificial warming of the specimen, but gentleness in handling and maintaining as far as possible at body temperature should be borne in mind. The laboratory should be ready for examination of the specimen when it arrives. The necessary implements, cleansed in plain hot water and thoroughly dried, should be kept ready for the examination. At the appointed hour, all the apparatus should be ready, including the microscope, to avoid thermal shock to the semen and the annoyance of misty lenses.

Gross examination

This examination includes a notation of the amount, which, when complete, and from men under 40 yr. of age, should exceed 3.5 ml. Lesser amounts should arouse suspicion of deficiency and premature weakening by vaginal acidity, otherwise negligible. Extended periods of sexual abstinence may impart a slightly yellow tinge to the normal grayish opaqueness. Normal specimens commonly show a slight degree of viscosity, which is easily demonstrated by slowly expelling a drop of semen from a pipet. Absence of viscosity with a lessening of opaqueness points to reduced cell content, although the reverse deduction does not hold.

An alkalinity of pH 8.1-8.4 has been so constant that it seems an unnecessary refinement except in research study; the significance of wider variations in reaction is not yet known. It has been pointed out also that the loss of carbon dioxide alters the body chemistry of the specimen.

Microscopic examination

Two specimens, one thick and one thin, are promptly prepared for microscopic examination by gently protecting a full drop and a fractional drop with cover glasses, the former permitting a gross impression of the density and motility of the cellular content and the latter allowing a preliminary study of individual cell morphology. In the thicker preparation, semen of relatively high fertility presents a field teeming with spermatozoa, many of which are rapidly propelled by fast-whipping tails. While universal cell activity would constitute the ideal, one usually notes in comparatively vigorous specimens that these fast travelers are colliding with a certain number of inactive (average 25%) and a variable number of sluggish and impotent cells, which gradually sink toward the lower strata of the field. Familiarity with this picture under standard dry high-power magnification constitutes the measure by which motility is judged. It is a practical observation that a motile field, though definitely subnormal, is apt to convey a favorable impression on the inexperienced or occasional examiner, but reverse errors in interpretation are rarely made. The practical aspects of motility are that a large number of highly motile cells are essential to fertility, and when a specimen of normal quantity and rich cellular content shows 25% or more of these dynamic cells, a rating of relative fertility must be assumed unless strongly disproved by other indices to be described. Such specimens usually meet other major requirements of fecundity.

Richness of cellular content, in terms of millions per milliliter, has been given emphasis by Macomber, Meeker, and other workers as a further index of appraisal. Although significant degrees of oligospermia are usually apparent to the experienced examiner on microscopic examination, there is no verbal picture that will adequately provide such an index. It may be said, however, that such specimens usually present other evidences of subnormality, such as lowered motility, increased number of abnormal cells, early crystal formation, and sometimes considerable indescribable debris. Such specimens may contain several round, granular cells in each field. These may be leukocytes or cells from the seminiferous tubules. The larger granular cells are spermatocytes; there are usually 4 nuclei, which may be demonstrated by the staining process later described. Cell counts are probably modified by the amount of the seminal fluid, and although in agreement with other observers that counts above 70,000,000/ml. are found in fertile specimens, Cary and Hotchkiss hesitate in setting a medium arbitrary count below which fertility is impossible. On the other hand, they have found a count as high as 112,000,000 in a highly defective specimen which measured less than 20 minims total quantity. This specimen promptly improved after treatment, and pregnancy followed.

Duration of motility is one essential to fecundation that can be easily determined. For this purpose the edge of the cover glass on the thicker smear should be protected by a layer of petrolatum, or new smears should be made from hour to hour from the total specimen. A normal specimen exhibits little cessation at the third hour after emission, and considerable cellular activity should be found at the fifth or sixth hour. Complete or considerable subsidence of motility within this limitation should be regarded as a further index of deficiency, but it should be noted that the few motile cells that were originally active in a highly defective specimen occasionally survive for many hours. Reciprocal studies between the direct male specimen and its behavior in female secretions tend to indicate that duration is practically synonymous with early vigor of motility, but there may be exceptions to this rule.

An improved staining process to define more clearly the exact morphology of the spermatozoon has been devised.

It has been found that Schaudinn solution is ideal for the fixation process, for it not only preserved the normal morphology of the cells but also removed the objectionable debris. The next problem was to devise a balanced stain that would demonstrate all portions of the spermatozoon, yet spare the chromophilic substance from overstain.

The method of Cary and Hotchkiss (p. 2072) permits illustration of the head, midsection, and tail of the sperm.

In appraising seminal specimens deduct the percentage of abnormal forms found from the total cell count. The resultant figure, when correlated with other data, is of great importance in the more accurate evaluation of the semen. For instance, 40% of inactive cells with

25% abnormal forms would indicate more serious impairment and need for greater improvement in a specimen with a count of 60,000,000 or less than would obtain with a cell count of 100,000,000.

Weisman method of counting spermatozoa

Weisman[4] uses a simple method which obviates the necessity of blood counting pipets and special diluting fluid. Dilution with ordinary tap water serves not only to dilute the sperm cells so that they may be easily counted in a Neubauer blood-counting chamber, but it also causes immediate cessation of motion of the sperms, facilitating the count.

Procedure:

1. Place 1 ml. well-mixed whole semen in an ordinary test tube, graduated at 20 ml. Add ordinary tap water, cold, up to the 20 ml. mark. This technic kills all the spermatozoa at once and by dilution of 1:20 diminishes the error which occurs when using the small amounts of semen usually utilized in the blood-counting pipet technic.
2. Fill the counting chamber. Count the sperms in the same fashion as white cells (p. 1105). Count and recheck the sperms in 1 sq.mm. (the 16 small squares in one corner of the chamber); multiply by 10 to obtain the number of sperms found in 1 c.mm. Multiply this by 1000 to find the number in 1 ml. diluted semen. To obtain the number in 1 ml. whole undiluted semen, multiply this by 20. In other words, multiply the actual average count in the 1 sq.mm. field by 2 and add 5 zeroes.

MEDICOLEGAL EXAMINATION OF SEMINAL STAINS

The identification of seminal stains is frequently of great value in medicolegal work in cases of alleged rape, sexual homicide, or even adultery. Specimens are sent to the laboratory often dried on clothing, sometimes admixed with blood, sometimes in dry masses adherent to hair. Spots on the specimen may be found very readily if submitted to examination by ultraviolet light. Seminal stains give a greenish white fluorescence which may aid in finding the stained areas. This fluorescence is not specific for semen.

Seminal stains are detected definitely by finding spermatozoa under the microscope. The spermatozoa are not readily destroyed unless the stain has been subjected to too much washing or exposure. After they become dry, they are very brittle, and the tail is easily separated from the head. To identify them absolutely, it is necessary to find perfect bodies with head and tail complete. It is best if motility can be shown (only possible when specimen is fresh and moist). Great care must be exercised in handling stains that are to be examined for the presence of spermatozoa.

If it is possible to obtain a fresh specimen, spermatozoa may be found by putting a small sample of the semen in a drop of physiologic saline and examining microscopically; motility may be found in recent specimens. The slide may be stained by various methods.

The spermatozoa, or male germ cells, are developed in the convoluted tubules of the testes. They are actively motile in the seminal fluid, which is a secretion of the accessory sexual glands, and are seen in enormous numbers in the normal ejaculate. A spermatozoon is a slender elongated flagellated structure 50-70μ in length and possesses a head, neck, a body or connective piece, and a tail.

The head is pear-shaped, measuring from 3-6μ in width. The neck consists of the anterior centrosome; the body is rodlike and connects the head and neck with the tail. The tail is divided in 2 portions, the chief piece and the end piece.

Spermatozoa are actively motile in the fresh state.

Seminal fluid or semen is a mixture of secretions of the epididymis, the ductus deferens, the seminal vesicles, the prostate, the bulbourethral or Cowper's glands, and the urethral or Littre's glands. Average amount of ejaculate is 2.5-5 ml., and each milliliter should contain 100,000,000 or more spermatozoa.

Methods of staining spermatozoa

Method of Cary-Hotchkiss[5] (1934)

Preparation of specimen:

1. Prepare thin cover-slip smears as used in the preparation of blood for staining.

Fixation in Schaudinn solution:

1. While still wet immerse for 1 min. in 7% corrosive mercuric chloride, 2 parts, and absolute alcohol, 1 part.
2. Immerse for ½ min. in 50% alcohol.
3. Immerse for ½ min. in distilled water, 3 oz., and tincture of iodine, 2 drops.
4. Wash in tap water.

Staining process:

1. Immerse for ½ min. in 5% aqueous solution of eosin.
2. Immerse for 1 min. in 50% alcohol, 3 oz., and concentrated hydrochloric acid, 2 drops.
3. Wash in distilled water.
4. Immerse for 2½ min. in hematoxylin.
5. Immerse for 1 min. in distilled water, 3 oz., and glacial acetic acid, 2 drops.
6. Wash in distilled water. Dry and mount.

This method gives excellent contrast between the nuclear material, which stains blue, and the cytoplasm, which stains red.

Method of Meaker[6]

1. Fix with heat.
2. Wash with 0.5% Chlorazene to remove mucus.

3. Wash with water.
4. Wash with 95% alcohol and dry.
5. Stain with carbolfuchsin and bluish eosin (carbolfuchsin, 2 parts, filtered bluish eosin, 1 part, alcohol, 1 part).
6. Wash with water.
7. Stain with methylene blue (methylene blue, 1 part; water four parts).
8. Wash with water.

Method of Gelarie[7]

This is a 1 min. method for the staining of spirochetes, spirilla, spermatozoa, and related organisms.

Preparation of solutions:

1. Fixing solution.
    (a) Dissolve 2.5 gm. recrystallized zirconyl chloride ($ZrOCl_2$) in 100 ml. 10% sodium chloride solution.
2. Citric acid solution.
    (a) Make 10% solution of recrystallized citric acid in distilled water to which a few crystals of thymol are added to prevent the growth of fungi.
3. Staining solution.
    (a) Dissolve 0.25 gm. gentian violet in 100 ml. N/10 solution sodium lactate; pH of this mixture should be approximately 8.4; if not, adjust.
4. K_2HgI_4 solution.
    (a) Dissolve 0.5 gm. mercuric iodide in 100 ml. 0.5% potassium iodide solution.
5. Counterstain.
    (a) Dissolve 0.2 gm. methylene blue in 100 ml. distilled water and add 0.2 gm. phenol (for photographic purposes use aqueous carbolfuchsin instead of methylene blue).

Procedure: According to Gelarie, the material to be examined is smeared on the slide in a thin layer and allowed to dry in the air (not over the flame).

1. Cover with the zirconyl chloride solution, allow to stand for 5 sec., and then wash with water. This acts as a fixative.
2. Then place a few drops of the 10% citric acid solution on the smear, allow to remain for 10 sec., and wash again with water. The slide is now ready for staining.
3. Place a few drops of the staining solution on the slide, allow to remain for 30 sec., and then wash off with water.
4. Follow by momentarily covering the smear with a few drops of the complex mercuric iodide solution and again wash with water. While a good stain is obtained at this point, uniformly excellent results may be secured by counterstaining with aqueous carbol methylene blue solution for 5 sec.
5. Then wash the slide, dry, and examine by means of the oil immersion objective.

Counterstaining is not recommended for obtaining exact detail of spermatozoa, however, due to overstaining of the organism. Without it the different parts—head, neck, and tail—with their components can be readily distinguished.

Although the above technic is simple, it is believed that some of the more complicated stains show up much more detail of the spermatozoal head.

Method of Holbert[8]

The Holbert method is similar to that used by Williams.

1. Make a thin smear like a blood film.
2. Shake until dry.
3. Flame gently.
4. Flood slide with 0.5% Chlorazene for 2 min.
5. Wash gently with running water.
6. Flood slide with 95% alcohol and let stand for 1 min.
7. Tip off excess alcohol and allow to dry.
8. Stain with 0.5% aqueous gentian violet for 3 min.
9. Rinse with water.
10. Rinse with 95% alcohol.
11. Rinse with water.
12. Counterstain with 1% aqueous rose bengal for 1 min.
13. Wash with water.
14. Allow to dry.

This method is recommended.

Methods of Williams et al.[9, 10]

Williams has 3 methods of staining spermatozoa. The smear is made in the same way as a blood film, dried in the air, fixed with moderate heat, and treated with 0.5% Chlorazene solution for 2-4 min. to remove the mucous film that overlies the spermatozoa. Wash slide gently with distilled water and then with 95% alcohol. Stain as follows.

Method I—crystal violet and rose bengal:

1. Cover film with 0.25% aqueous solution crystal gentian violet for 2-3 min.
2. Wash with water.
3. Decolorize with 95% alcohol for ½-2 min.
4. Wash with distilled water.
5. Counterstain with 1% rose bengal (aqueous) for 8 sec.
6. Wash in water and examine under microscope.

If the spermatozoa have not taken up sufficient rose bengal to impart a distinct reddish color to the acrosome and the nuclear membrane, repeat the staining interval with rose bengal until this is accomplished. Care should be taken not to overstain with rose bengal; if overstained, the contrast between the nucleus and the acrosome will be lost.

Method II—Wollschwarz, methylene blue, and rose bengal: Stain in 1% solution of Wollschwarz for 5 min. as follows, preparing 3 solutions.

1. Solution A.

Wollschwarz, 1% aqueous .	4 ml.
Sulfuric acid, 2%	0.025 ml.

2. Solution B.

Wollschwarz, 1% aqueous .	4 ml.
Sulfuric acid, 2%	0.05 ml.

3. Solution C.

Wollschwarz, 1% aqueous .	4 ml.
Sulfuric acid, 2%	0.075 ml.

The smear on the microscope slide is marked off into 3 divisions with a glass-marking pencil, and the solutions of stain having the different concentrations of sulfuric acid are applied to each of the divisions, respectively (dilutions A and C may be omitted).

1. Wash with water.
2. Counterstain for 6-8 sec. with Loeffler methylene blue that has been diluted in 15 parts

of water. This acts as a mordant to the Wollschwarz, serving to fix it in the nucleus. It imparts a rather greenish hue to the otherwise yellowish-stained nucleus.
3. Wash with distilled water.
4. Counterstain with 1% aqueous rose bengal for 6 sec. or until the rose bengal is set in the acrosome and the nuclear and cell membranes to give the desired contrasts.
5. Examine slide after each application of stain so as to guide its intensity.
6. Wash with distilled water.
7. Dry, mount in neutral Canada balsam, and apply cover slip or examine without mounting.

Method III—carbolfuchsin, eosin-B, and methylene blue: Prepare the following 2 staining solutions.

1. Stain 1.

Saturated alcoholic solution of eosin (bluish)	1 ml.
Ziehl-Neelsen carbolfuchsin . .	2 ml.
Alcohol, 95%	1 ml.

Mix the eosin and carbolfuchsin solutions together in a dry watch glass. Immediately on mixing, an insoluble precipitate and a metallic film form. Filter the mixture, and to the filtrate add 1 ml. 95% alcohol.

2. Stain 2.

Loeffler methylene blue . .	1 part
Distilled water	6-8 parts

Apply Stain 1 to the film for 2 min. Wash film gently with distilled water. This is best done by immersing it in a basin. Counterstain with Stain 2 for 4-8 sec. Wash slide with distilled water and dry.

Method of Pollák-Joël[11]: Use 2 separate films and stain them differently. One stain brings out the structure of the entire spermatozoon and the second affords better visualization of the cell structure. Thus the stains complement each other.

1. Stain I—hemalum-eosin (for visualizing spermatozoal structure).
   (a) Fix for 2 min. with methyl alcohol and then rinse in water.
   (b) Stain for 20 min. with hemalum.*
   (c) Steep in running water for 30 min. with fresh hemalum, rinse for 10 min. in water, stain for contrast for 3 min. with 3% alcoholic eosin solution.
   (d) Differentiate in an alcohol sequence of 70, 90, and 100%.
   (e) Clear in carbolxylene and embed in Canada balsam under a cover glass.

2. Stain II—azure-eosin (for the cellular structure).
   (a) Fix for 2 min. with methyl alcohol.
   (b) Stain for 20 min. with a diluted Giemsa stain.
   (c) Rinse in distilled water and embed in Canada balsam under a cover glass.

The staining solution is prepared fresh each time; to 5 ml. distilled water add 10 drops of Giemsa stain.

Examination for spermatozoa in seminal stains

If the stain is such that a fragment can be removed with the point of a knife, the examination is fairly easy. Remove a portion of the scaly stain with a knife point and place it on a glass slide on which has previously been placed a drop of distilled water. Allow the stain to soak in the water for several hours. Keep the specimen moist under a bell jar or in a large closed Petri dish. Separate the scales very cautiously with a teasing needle, allow to dry, fix in methyl alcohol, and stain with dilute Giemsa stain (1 drop in each ml. neutral distilled water) for 30 min., repeating the stain for an additional 30 min. if necessary. If restaining is attempted, dilute fresh stain. Wash gently with distilled water, stand the slide on end, and allow to dry. Examine for spermatozoa under the oil immersion objective.

If the stain is on unstarched cotton or linen cloth the finding of spermatozoa presents a much more difficult problem. Select a thread of the cloth near the center of the stain; select the side of the cloth which contains the more seminal fluid. Place a thread from the dry stain on a glass slide in a drop of distilled water. Make many such preparations. Each specimen is allowed to soak in the water for 2-24 hr. Provision must be made to prevent drying of the water during this time.

At the end of the digestion period tease the fibrils apart **very cautiously** with fine pointed needles. Take care not to break the spermatozoa. Continue with the staining process just described.

The cloth itself may be stained according to the **method of Baecchi.**[12] A piece of the cloth containing the stain is cut 1 cm. square and placed in 1% acid fuchsin or methylene blue solution. It is then washed in 1% hydrochloric acid, after which it is dried in the air or dehydrated in absolute alcohol. Next it is cleared in xylol and then mounted in Canada balsam. If the spots are not fresh, they are placed for ½-24 hr. in 20-30% ammonium hydroxide solution and then in distilled water preliminary to the staining process.

Hektoen and Rukstinat[13] describe a **method** of making a stained specimen from spots of spermatozoa on cloth. Their method makes use of Mallory-Wright chloride of iron-hematoxylin stain.

1. Soak the stained cloth in 1% ammonium hydroxide solution in water for 12-24 hr.
2. Select a single thread and stain 2 min. in 5% aqueous solution of erythrosin.
3. Wash quickly in distilled water.
4. Stain with 10% aqueous solution of ferric chloride 2 min.
5. Transfer directly to 2% aqueous solution of hematoxylin for 2 min.
6. Wash quickly in distilled water.
7. Transfer to 0.5% aqueous ferric chloride and tease out fiber to its finest shreds. Remove from this solution when threads are reddish pink or light brown.
8. Wash in distilled water quickly.
9. Dehydrate in absolute alcohol.
10. Clear in creosote.
11. Mount in balsam.

Better results are obtained if the differentiation is carried out slowly in 0.5% ferric chloride solution than in stronger solutions. Boiling the cloth for 1 min. in water after soaking in ammonia solution helps preserve the tails. When the stain takes well, the head of the spermatozoon is stained dark purple to black in the posterior two thirds, whereas the anterior third is pink, and the tail is pink to lavender. The connecting piece frequently is evident. The threads of the cloth may stain more or less pinkish.

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*Hemalum is similar to hematoxylin but not identical. It is a 2% solution of crystalline hematin in 95% aqueous solution of $KAl(SO_4)_2 \cdot 12 H_2O$, 100 ml.

Weisman[14] refers directly to the technic of Hankin[15] and Windsor.[16] This is a special technic for warm climates. Hankin examined the suspected stains directly on the cloth, after first fixing the material with a cellular fixative, then adding dilute sulfuric acid, and finally staining the material with gentian violet. Windsor fixed the cloth in Müller solution and then stained it. Williams suggested the special Wollschwarz stain. Soak and scrape the semen-stained cloth, obtaining a turbid suspension. Centrifuge the soakings. In an old specimen some of the tails will be missing or bent or abnormally twisted. Care must be taken not to confuse the sperm heads with spores, pus cells, or artifacts.

Florence test for identification of semen

Reagent:

Potassium iodide	1.65 gm.
Iodine	2.54 gm.
Distilled water	30 ml.

Rub up the iodine and the potassium iodide in a mortar and then add the water. Filter before using.

Procedure:

1. Remove a minute fragment of the stained specimen by means of a pair of fine-pointed forceps and sharp-pointed scissors.
2. Transfer to a slide.
3. Treat with a small drop of distilled water.
4. Allow to soak for 1-2 min.
5. Add a small drop of the reagent to it in such a way that the 2 drops of fluid come in contact at their edges and then cover immediately with a cover glass.
6. Examine microscopically for seminal crystals. These look like the hemin crystals obtained with the Teichmann test for blood (Fig. 681).

Weisman states that a positive reaction is not significant since other body fluids give such results with this test. Our own experience confirms this statement. A negative test, however, informs the investigator that the suspected substance is not semen, and this is the only value of the Florence test.

Peltzer[13] uses the following technic for the Florence method:

1. Moisten the suspected spots with hydrogen peroxide. If semen is present, much foaming occurs.
2. Centrifuge substance.
3. Place on slide and stain with 2% aqueous eosin.

If positive, long lancelike crystals appear. The addition of I-KI solution to the slide colors the crystals brown. The crystals disappear and are recrystallized by the addition of more I-KI solution. The presence of Florence crystals denotes the presence of body fluids, possibly semen.

Puranen test

Puranen[18, 19] and Tarsitano[20] advocated a microchemical method for identification of spermatozoa. Naphthol yellow sulfur in aqueous solution reacts differently with human semen at neutral and slightly alkaline reactions. A positive reaction is denoted by formation of microcrystals, which are characteristically large, have a strong double refraction, and are of a definite orange hue.

Niederland test[21]

This test employs dilute sulfuric acid. When added to a suspected specimen, the test is always positive in the presence of semen, but this test is also positive with practically all other body secretions. Weisman states that its only value lies in the fact that, when the test is negative, further search for spermatozoa can be abandoned. The crystals formed are probably sulfates of calcium and other salts found in semen and body fluids.

Marcille method[22]

This is nothing more than the Florence test coupled with the addition of Gram-eosin staining of the centrifuged precipitate after maceration of the specimen in physiologic saline solution.

Borgarius test

This test is similar to the Florence test, except that phosphomolybdic acid solution instead of the I-KI solution is used to precipitate crystals. The crystals obtained by this technic are characteristic, but they are also found with other body fluids.

AuI_3 (auric iodide) test

Villamil[23] has shown that solutions of AuI_3 give characteristic crystals with diluted semen as well as with solutions of spots and stains produced by semen. The crystals are distinct. He considers this reaction to be more sensitive than that obtained by most other microcrystalline tests.

Test for hydrogen ion concentration[24]

If a drop of distilled water is added to suspected semen and well mixed, the hydrogen ion concentration of the solution when tested with Nitrazine paper and compared with a colorimetric chart will give evidence of definite alkalinity.

Fluorescence or ultraviolet light test

Ito[25] first demonstrated that semen stains give a characteristic color effect under ultraviolet light. He obtained this same effect with other tissues and described its use in forensic medicine.

Simonin[26] showed that dried sperm gives a brilliant characteristic fluorescence under ultraviolet light but stated that other organic liquids also give some fluorescence, so that the test is probably not specific. Husson[27] reported that seminal spots, irrespective of spermatozoa or azoospermia or the age of the spot, show a characteristic fluorescence in ultraviolet rays between 4200 and 4900 Å.

Microchemical identification of seminal fluid—acid phosphatase method

Determination of acid phosphatase activity affords an excellent means for specific microchemical identification of seminal fluid and seminal stains. This procedure employs a principle postulated by Ove Riisfelt of Denmark in 1946.[28] It is of definite medicolegal value in the identification of seminal fluid as an adjunct to the demonstration of spermatozoa, but it is of particular value in cases in which spermatozoa cannot be demonstrated in the specimen.

Kaye found that male ejaculate produces a very high acid phosphatase activity in comparison with the very low acid phosphatase activity of the other body fluids (saliva, perspiration, urine, etc.) and the common vegetable and fruit juice stains. The differences are so marked and definite that it leaves little room for doubtful interpretation of the results. The average seminal fluid contains approximately 2500 King-Armstrong units of acid phosphatase/ml., the upper limits ranging to 3500 King-Armstrong units and the lower to above 500 units. Ejaculates in cases of aspermia give comparable high results.

Semen is characteristic in that it gives these high values, whereas other body fluids give values of much less than 5 King-Armstrong units/ml. The enzyme is resistant to all known body fluids including vaginal secretion; it might, however, be inhibited by vaginal disinfectants and contraceptives. Seminal fluid aged 2 wk. in a condom-type contraceptive showed no appreciable loss of acid phosphatase activity. Known seminal stains proved to be strongly positive even after many months, 6 or more.

The materials listed in the accompanying outline contain less than 5 King-Armstrong units of acid phosphatase/ml. concentrated pure substance and in the cases of vaginal fluids, perspiration, feces, nasal discharge, and pus, per unit sq.cm. saturated control cloth.[29]

I. Body fluids
    Vaginal fluids
    Urine
    Blood (serum) (in carcinoma of prostate with metastases, this value may be appreciably increased)
    Blood (whole)
    Blood (menstrual)
    Saliva
    Perspiration
    Pus
    Nasal discharge
    Gastric juice
    Feces
    Rectal smears
II. Food stains
    Tea
    Coffee
    Egg white
    Egg yolk
    Vegetable juice (V-8)
    Orange juice
    Prune juice
    Beer
    Milk
    Cream
    Mushrooms
    Corn
    Alkaloids, such as quinine, atropine, and procaine
    Meat gravy
    French dressing
    Beet juice
    Custard
    Starch
    Mayonnaise
    Chop suey
    Soapy water (Ivory)
    Onions
    Albumin
    Choline

Walker acid phosphatase test for identification of seminal fluid[30]

Principle: The portion of the garment to be tested is immersed in a bath at pH 5.0 containing alpha-naphthyl phosphate as a substrate and a suitable diazonium salt. The acid phosphatase of the semen acts on the phosphate ester to liberate alpha-naphthol. This immediately reacts with the diazonium salt to form a brilliant orange-red azo pigment, which is insoluble under the conditions of the test, and which becomes adherent to the background at the site of acid phosphatase activity.

Substrate-reagent bath:

1. Dissolve 23 gm. sodium chloride, 0.5 ml. glacial acetic acid, and 2 gm. sodium acetate trihydrate in 90 ml. distilled water.
2. Add 1 ml. 1% aerosol containing a suspension of 30 mg. anthraquinone-1-diazonium chloride (III) and 50 mg. calcium-1-naphthyl phosphate.
3. Dissolve the aerosol in warm water without shaking.

Preparation of anthraquinone-1-diazonium chloride (III):

1. Dissolve 10 gm. 1-amine anthraquinone (Eastman technical grade) in 85% sulfuric acid and pour into a large volume of water. Collect the precipitate with suction and wash free from acid with water.

2. Treat the wet product with 20 ml. concentrated hydrochloric acid to form a grayish white crystalline sludge.
3. Diazotize at 30°-40° C. by running an equivalent of sodium nitrite, 3.2 gm. in 50 ml. water under the surface with stirring.
4. Pour the reaction mixture into 10 times its volume of water at 70°-80° C.
5. Add 0.5 gm. sodium nitrite.
6. Filter rapidly.
7. Add sodium chloride to salt out the diazonium chloride in shiny, yellow platelets.
8. Collect the crystals, wash with cold water, alcohol, and ether, and dry.
9. Protect from light and store at room temperature, or in a refrigerator. Refrigerated they are stable at least 18 mo.

The diazonium compound is available commercially as Naphthanil Diazo Red AL. It contains 5% zinc chloride and 12% aluminum sulfate. The metal ions completely inhibit acid phosphatase present in the cell nuclei of liver and kidney, whereas the cytoplasmic acid phosphatase is altered slightly. This finding is suggestive of a third variety of acid phosphatase present in cell nuclei.

Preparation of calcium alpha- or beta-naphthyl phosphate*:

1. Dissolve 14.4 gm. alpha- or beta-naphthol in 15 ml. dry pyridine.*
2. Dissolve 15.3 gm. phosphorus oxychloride solution in 10 ml. dry pyridine.
3. Add the phosphorus oxychloride solution drop by drop, or the entire material will be lost.
4. Add the naphthol solution in portions to the phosphorus oxychloride solution. Heat is developed, and a white precipitate separates as a sludge.
5. Allow to stand at room temperature for 10 min.
6. Cool and add in portions to a solution of 30 gm. calcium acetate in 200 ml. distilled water and 100 gm. ice. An oily material separates which slowly dissolves and comes out as a white precipitate.
7. Add 150 ml. alcohol.
8. Collect the white precipitate with the aid of suction.
9. Wash with 95% alcohol and ether and dry.

Procedure:

Immerse the garment to be tested in a substrate reagent bath. If acid phosphatase is present, a brilliant orange-red pigment will appear and adhere to the background at the site of acid phosphatase activity. The phosphatase activity of a fresh, dry seminal stain is so intense that within 30 sec. a distinctive, visible coloration occurs, and within 2 min. the test is complete.

When the semen is mixed with blood, as occurs in rape, an interesting color change occurs. The blood turns to a dirty brownish gray when immersed in the substrate bath. Wherever semen is present, this color is gradually replaced by the orange red of the phosphatase-catalyzed pigment. Bloody stains should be developed longer than others, so that the pigment formed by the semen may be visible above the background.

If the suspected stain occurs on an unfavorable background, such as a red wool dress, the red pigment of the developed test is not clearly visible. In this case, and in any case in which it is not desirable to apply the reagents directly to the specimen, a portion of the phosphatase of the stain may be transferred to a white filter paper, and its activity can be detected thereon.

Moisten the filter paper, press it firmly against the suspected area for several minutes, and then immerse the paper in the substrate-reagent bath. Some of the acid phosphatase of the stain on the garment will have diffused into the filter paper sufficiently to cause a print that can be developed.

If desired, place a small portion of the suspected material on dry filter paper. Moisten with a drop of water. Development of the paper will show a red rosette if semen is present.

Even if the fabric is tested directly with the substrate-reagent mixture, the procedure does not interfere with the subsequent microscopic search for spermatozoa. The seminal fluid takes on a peach-pink color, but the spermatozoa are not colored, for they do not contain sufficient acid phosphatase. If the specimen is then stained with carbolfuchsin, the spermatozoa stain with the dye. The test serves the double function of demonstrating high acid phosphatase activity and at the same time indicating where a search for spermatozoa is likely to be successful. The phosphatase activity is largely concentrated at the periphery of the stain. Spermatozoa are more plentiful in the central area, where they may be demonstrated microscopically.

Phosphatabs-Acid* may be used in place of the Walker test.

1. Cut cloth ¼ in. × ½ in., put in a 50 ml. beaker, and add 10 ml. distilled water.
2. After 30 min. remove the cloth, stir the liquid in the beaker, and allow the particles to settle.
3. Place 4 drops of the clear supernatant liquid in one of the wells in the Warner-Chilcott kit.
4. Add a silver foil tablet, crush with a glass rod, and allow to remain at 20° C. for 22 min. (time varies with temperature).
5. Add a gold foil tablet and crush.

An immediate dark purple color indicates a high acid phosphatase content.

Precipitin test for identification of human seminal stains

Precipitin tests for the differentiation of semen of human and animal origin can be made by carrying out the test described below.

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*Reagent pyridine contains enough water to decompose the phosphorus oxychloride extensively. Dry by refluxing with anhydrous barium carbonate for several days, distill, and store with sodium hydroxide pellets.

*Warner-Lambert, Morris Plains, N. J.

In the examination of stains in cases of suspected rape, finding spermatozoa is by far the most conclusive evidence of the presence of semen. The procedure just described for the identification of seminal fluid, however, is of value in proof of the presence of semen especially in cases of aspermia or in those cases in which complete spermatozoa cannot be identified (twisted tails, etc.).

The injection of rabbits with the semen of man, swine, bull, or horse induces the formation of specific precipitins for the homologous fluid.[31-36] In most cases precipitins develop also for the homologous blood serum, as already known. Extracts of human spermatozoa in salt solution appear to give the same result as the seminal fluid. Seminal precipitin reaction reveals the presence in semen of a substance or substances peculiar to it, and consequently the reaction may be of value in studying the nature of suspected spots and stains.

Precipitin for identification of human semen is made by injecting rabbits intramuscularly 4 or 5 times at intervals of 3-4 days with increasing quantities of human seminal fluid. The first injection is 2 ml., the next 4 ml., each successive injection being 2 ml. more than the preceding one. Six or 8 days after the last injection the animals are bled, and the blood is collected in sterile 50 ml. Rockefeller tubes with cork stoppers. After the blood has clotted, the clot is shaken loose, and the tube is centrifuged at 1500 r.p.m. for 20 min. to separate the serum. The serum is removed with all sterile precautions and placed in another sterile test tube. Serum precipitates are removed from the rabbit's blood serum by mixing it with equal parts of a 1:200 dilution of human blood serum in saline. Allow the mixture to stand at room temperature for 1 hr. and then place in a refrigerator overnight. Centrifuge it at high speed for sufficient time to sediment the precipitate formed from the mixture of the rabbit and human serums. Use the supernatant serum for precipitin tests to identify seminal fluid stains.

Procedure:

1. Extract the suspected seminal stain in physiologic saline for 4-8 hr., using very small amounts of saline.
2. Concentrate the extract slightly and then centrifuge to remove any sediment that may be present.
3. Remove the supernatant fluid, placing it in a clean, dry test tube.
4. Dilute with physiologic saline until, upon shaking, the amount of foam indicates a 1:1000 dilution. Be sure the diluted extract is neutral.
5. Place 0.1 ml. antisemen precipitin serum in the bottom of a small heavy walled capillary tube.
6. Allow the extract of suspected seminal fluid stain to run down the side of the tube so that it forms a layer above the serum but does not mix with it.
7. Allow to stand at room temperature for 1 hr., observing the reaction at 10-15 min. intervals.
8. At the same time run a control test using an extract of an unstained portion of the cloth on which the seminal stain was found.

A positive reaction is indicated by a white precipitate at the point of contact between the 2 fluids. This reaction is like the reaction in the precipitin test for the identification of human blood (pp. 1613ff).

EVIDENCE IN SUSPECTED SEXUAL ASSAULT

In rape of a child or virgin there is usually ample evidence in the form of hemorrhage and laceration of tissue. Further evidence may be obtained by looking for traces of semen either on the external portions or in the vagina. The length of time that spermatozoa may be found in the vagina after an alleged assault is varied, some reporting that spermatozoa are found as long as 2 days after intercourse. Although this is true in rape cases without death from the assault, in those cases in which death has occurred, spermatozoa apparently may not live very long in the vagina.

In the assault cases spermatozoa may be found on the clothing by one or more of the tests already described. In addition to the examination of the victim and her clothing, the clothing should be further examined for fibers of the assailant's apparel. One should also examine the clothing of the victim and suspected persons for evidence such as grass, fibers, soil, etc., which may be related to the locality where the crime was committed. In many cases there will be found scratches and skin changes due to the fingernails of the assailant or the victim. The suspected person should be carefully examined, particularly for skin, hair, and cosmetics, which may be identified as coming from the victim. The scrapings of the nails of the victim should also be examined.

The technic of examination of the victim's vagina for spermatozoa is as follows:

1. Gently spray physiologic saline solution over the vagina and perineum of the victim, collect the washings in a clean dish placed under the patient, and then examine.
2. Gently wash the vaginal wall with a small amount of saline solution injected into the vagina with a clean glass syringe and rubber bulb such as is used in blood cell washing in serologic work.
3. Withdraw the contents of the vagina with the same syringe or pipet and after centrifugation, examine microscopically.
4. Also wash the suspect with saline solution sprayed over the entire penis, collect, centrifuge, and examine the sediment microscopically.

Identification of blood group of semen is described on p. 1543.

Chapter 117

Chemical analysis of water and milk

CHEMICAL ANALYSIS OF WATER

For the chemical analysis of water, a liberal sample of fresh water should be submitted to the laboratory. This sample, at least 1 gal., should be representative of the entire water supply which is to be examined. Procedures described here are largely those recommended by the United States Army and the American Public Health Association.*

Most of the equivalents in water chemistry calculations are computed on a parts per million basis. Suggestions for interpreting the quality of the water are given in Table 369.

Turbidity

Turbidity of water is due to suspended matter in the water. To determine turbidity of the specimen, compare with standard suspensions or standard disks ranging from 5 to 100 p.p.m. Report turbidity as less than 5 p.p.m. or between 10 and 15 p.p.m. or as 5 p.p.m., etc.

Standard suspensions:

1. Place 5 gm. Fuller's earth in 1 L. distilled water.
2. Agitate intermittently for 1 hr. and allow to stand for 24 hr.
3. Withdraw the supernatant liquid without disturbing the sediment.
4. Dilute successive portions of the sediment with distilled water to prepare the various standards ranging from 5-100 p.p.m. Use a candle turbidimeter for standardization.

Standard disks: Permanent turbidity standard disks are available from most large chemical supply houses. The disks may be used in place of the turbidity standard suspensions.

Combination water color and turbidity test sets, United States Geological Survey Standard, may be purchased from any large chemical supply company.

Procedure: The standard unit of turbidity is that produced by 1 p.p.m. silica in distilled water.

1. Thoroughly shake the water to be tested. Pour a portion of it into a 125 ml. glass-stoppered bottle and shake.
2. Shake the standard suspensions and compare in a dark box turbidimeter. Record turbidity in p.p.m. Permanent standard disks may be used in place of the standard suspensions. If the Jackson candle method is used, report in turbidity units.

Color

Color disks, calibrated from a platinum-cobalt color scale, may be purchased from any large chemical supply house.

1. Place the glass disks on the end of a metal tube containing distilled water.
2. Place the water to be tested in a second tube.
3. Compare and record the color number.

Odor

Odor of water specimens may be described as musty, aromatic, fishy, moldy, etc., and as negative, faint, moderate, and strong. If an odor is detectable in the sample, the odor concentration must be determined.

1. Place 200 ml. sample in a 500 ml. flask.
2. Add 200 ml. water free of odor. This is a 1:2 dilution.
3. Mix without unduly exposing to the air. Do not agitate unnecessarily.
4. Add 200 ml. this dilution to 200 ml. water free of odor in another 500 ml. flask. This is a 1:4 dilution.
5. Mix and continue with the dilutions to obtain 1:8, 1:16, 1:32, and further dilutions, until the second dilution beyond which the odor of the specimen is no longer detectable. If the odor is very strong, omit some of the lower dilutions.
6. Shake and examine each flask in order, beginning with the strongest dilution and proceeding to the weakest until an odor is just detectable in one of the flasks. This is the

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*Standard methods for the examination of water and waste water, ed. 11, 1960, American Public Health Association.

threshold odor and is recorded as the odor concentration.

7. Report the **odor concentration** as the reciprocal of the dilution; e.g., 1, 2, 4, 8.

Hydrogen ion concentration

The determination of pH may be done with pHydrion or Nitrazine test papers or with a pH meter.

Total residue—loss on ignition

1. Shake the sample thoroughly but do not filter.
2. Place 100 ml. of sample in a weighed platinum dish.
3. Evaporate on a water or steam bath.
4. Dry the residue at 103°-105° C. for 1 hr.
5. Weigh the crucible and contents. Subtract the weight of the platinum dish from the total weight to obtain the total solids or residue on evaporation. This gives the residue at 103° C.

Loss on ignition and fixed residue:

1. Ignite the residue in the platinum dish at low red heat. Note any significant change in color or odor.
2. Allow the dish to cool.
3. Moisten the residue with distilled water, then dry at 103° C., cool, and weigh.
4. Subtract this figure from the weight recorded as "residue at 103° C." This figure represents the **loss on ignition.** The weight of the residue is recorded as the **fixed residue.**

Alkalinity

Reagents:

1. Phenolphthalein indicator.
    - (a) Dissolve 5 gm. phenolphthalein in 1 L. 50% alcohol.
    - (b) Neutralize with N/50 sodium hydroxide.

The alcohol should be diluted with distilled water which has been boiled and cooled quickly.

2. Methyl orange indicator.
    - (a) Dissolve 5 gm. methyl orange in 1 L. distilled water. Keep the solution in the dark.

Procedure with phenolphthalein:

1. Add 4 drops phenolphthalein indicator to 50 or 100 ml. water sample in a white porcelain casserole or in an Erlenmeyer flask over a white surface. If the solution becomes colored, hydroxide or normal carbonate is present.
2. Titrate with N/50 sulfuric acid from a buret until the color disappears. This is the end point.

Calculation: Ml. N/50 sulfuric acid used × 20 = phenolphthalein alkalinity in p.p.m. calcium carbonate if 50 ml. of water sample were used. If 100 ml. were used, multiply by 10 instead of by 20.

Procedure with methyl orange:

1. Use 2 drops methyl orange indicator to 50 or 100 ml. water sample. The solution develops a yellow color in the presence of hydroxide, normal carbonate, or bicarbonate.
2. Titrate with N/50 sulfuric acid to the first faint pink color, i.e., until the yellow color is no longer a pure yellow.

Calculate as for phenolphthalein.

Total hardness (soap method)

Reagents:

1. Stock standard soap solution.
    - (a) Dissolve 100 gm. pure powdered castile soap in 1 L. 80% alcohol.
    - (b) Allow to stand overnight.
    - (c) Decant.

This solution is approximately 10 times stronger than needed for the working solution.

2. Working soap solution.
    - (a) Titrate the standard soap solution against a standard calcium chloride solution, 1 ml. of which is equivalent to 1 mg. calcium carbonate.
    - (b) Dilute the stock standard solution with 80% alcohol in accordance with the titer so that 1 ml. is equivalent to 1 ml. standard calcium chloride solution.
3. Standard calcium chloride solution.
    - (a) Dissolve 0.5 gm. pure calcite with a small amount of dilute hydrochloride acid.
    - (b) Wash down with carbon dioxide-free distilled water and neutralize with ammonium hydroxide.
    - (c) Add a small amount of ammonium hydroxide to produce slight alkalinity.
    - (d) Dilute with carbon dioxide-free distilled water to 500 ml.; 1 ml. is equivalent to 1 mg. of calcium carbonate.

Procedure:

1. Place 50 ml. water to be examined in an 8 oz. glass-stoppered bottle.
2. Add from a buret the working standard soap solution, using small amounts (0.5 ml.) at a time.
3. Shake vigorously after each addition.
4. Continue adding the soap solution until a strong permanent lather, which stands 5 min. with the bottle laid on its side, is secured. This is the end point. When approaching the end point, reduce the addition of the soap solution to 0.1-0.2 ml. at a time.

A blank titration should be made using distilled water against the soap solution. The result of this titration is referred to as the **lather factor.**

Calculations: Subtract the lather factor from the titration results and multiply by 20 to obtain the total hardness in terms of calcium carbonate in p.p.m.

If a hardness test requires more than 15 ml. soap solution, dilute an aliquot of the water to 50 ml. with carbon dioxide-free distilled water so that the final lather point will be less than 15 ml. Deduct the lather factor (blank) and multiply the result by 20 and then by the dilution factor to obtain the correct hardness value.

If the water is acid, render it neutral to methyl orange using N/50 sodium carbonate solution before determining the total hardness.

Total iron

Reagents:

1. Standard ferric iron solution (1 ml. = 0.1 mg. Fe).
    - (a) Dissolve 0.7022 gm. of crystallized ferrous ammonium sulfate ($FeSO_4(NH_4)_2SO_4 \cdot 6H_2O$) in 50 ml. distilled water and 20 ml. concentrated sulfuric acid.
    - (b) Warm the solution and add potassium

permanganate solution until the iron is completely oxidized. Dilute to 1 L. with distilled water.

2. Dilute hydrochloric acid.
   (a) To 3 vol. distilled water add 1 vol. concentrated hydrochloric acid, specific gravity 1.2. This is an approximate 3N solution.
3. Potassium permanganate solution.
   (a) Dissolve 6.3 gm. of potassium permanganate in distilled water in a liter volumetric flask and dilute to 1 L. with distilled water. This is an approximate N/5 solution.
4. Potassium or ammonium thiocyanate.
   (a) Dissolve 2 gm. potassium or ammonium thiocyanate in distilled water in a 100 ml. volumetric flask and dilute to 100 ml. with distilled water.
5. Hydrochloric acid.
   (a) Concentrated hydrochloric acid free from iron.
6. Nitric acid.
   (a) Nitric acid, specific gravity 1.42, free from iron.
7. Nitric acid.
   (a) Nitric acid, specific gravity 1.195. Use 382 ml. this concentrated nitric acid/L. solution. This is approximately a 6N solution.

Procedure:

1. Evaporate 100 ml. (or less) of the sample to dryness. With silt-bearing samples of water the quantity of iron is sometimes so great that it is necessary to use as little as 10 ml. sample. In such cases add 5-10 ml. concentrated hydrochloric acid to the sample. If the sample contains much organic matter, destroy

Table 369. *Safe limits of chemical impurities in drinking water*

Impurity	*Desirable*	*Limit*
	Less than:	Not greater than:
Odor	None	Free from odor
Color	5	20
Turbidity	5	10
Ammonia nitrogen	0.04	0.9
Albuminoid nitrogen	0.05	0.1
Nitrite nitrogen	None	0.7
Nitrate nitrogen		0.5
Oxygen consuming power	1	2
Chlorides	50	250
Free residual chlorine	At least 0.2 (at tap)	Must not cause objectionable odor or taste
Total hardness (as $CaCO_3$)		250 10.2
Noncarbonate hardness	Relative to total hardness	
Acidity	Free mineral acids must be absent	
Total solids		500
Loss on ignition	Is a relative not a precise determination Represents not only organic matter but volatile decomposition products from carbonates, nitrates, and other components of residue	
Alkalinity (as $CaCO_3$)	It is desirable to have total alkalinity below 400 p.p.m.; however, alkalinity usually of no significance unless there is phenolphthalein alkalinity In case of nontreated waters, phenolphthalein alkalinity should not be greater than half of total alkalinity In chemically treated waters, phenolphthalein alkalinity (calculated as $CaCO_3$) should not be greater than 15 mg./L. plus 0.4 times total alkalinity; in case of waters softened by lime soda process, total alkalinity produced should not exceed hardness by more than 35 p.p.m. (calculated as $CaCO_3$)	
Minerals		
Silica		250
Magnesium	30	100
Calcium	Unimportant	50
Iron and manganese	0.1	0.3
Sulfates		250
Sodium and potassium	0	50
Aluminum		0.3
Toxic ions		
Arsenic	None	0.05
Lead	None	0.1
Copper	None	3.0
Zinc	None	15.0
Selenium	None	0.05
Barium	None	None
Molecularly dehydrated phosphates (chemical treatment)	Less than 10.0	10.0

the organic matter by ignition so that the iron dissolves only with difficulty.

2. Heat the dish to drive off excess of acid if the residue was not ignited.
3. Cool and add 0.8-1 ml. of 3N hydrochloric acid.
4. Warm on the water bath taking care to avoid evaporation to dryness by adding small quantities of distilled water from time to time.
5. Rinse the hot solution into a 50 ml. Nessler tube, filtering if necessary.
6. Add 1-2 drops of potassium permanganate solution. If the color of permanganate does not persist for at least 5 min., add more permanganate solution, drop by drop.
7. Cool and dilute to the mark with distilled water.
8. Prepare iron standards by placing in matched Nessler tubes 0.05 ml., 0.1 ml., etc., up to 4 ml. (or more) of standard iron solution covering the range of standards required for the determination at hand. Dilute each to about 40 ml. with distilled water.
9. Add 0.5 ml. 3N hydrochloric acid and 1 or 2 drops potassium permanganate solution to each tube. Dilute to the mark.
10. Add 5 ml. thiocyanate solution to the unknown and to the standards. Mix each tube thoroughly and compare immediately.
11. If the sample is low in organic matter, boil 50 ml. with 5 ml. 6N nitric acid for 5 min., add 3 drops of permanganate solution, and cool. Add 5 ml. thiocyanate solution and compare immediately with standards prepared from the standard iron solution with 6N nitric acid instead of with 3N hydrochloric acid.

Silica—Gravimetric method

1. Evaporate 500 ml. sample in a platinum crucible. When the residue is nearly dry add 1 ml. dilute hydrochloric acid (1:2), moisten the sides of the dish, and evaporate to dryness. If much organic matter is present, char in a radiator.
2. Add a few drops hydrochloric acid, dissolve in hot water, and filter.
3. Ignite and weigh the insoluble residue.
4. Add 2 drops concentrated sulfuric acid and a little hydrofluoric acid, volatilize the acids, ignite, and weigh again.
5. Report the loss in weight as silica (SiO_2). A weight of nonvolatile matter exceeding 0.5 mg. should be analyzed.

Interpretation of chemical analysis of drinking water

In Table 369 are given the ordinarily considered safe limits of impurities in drinking water of good quality from a **chemical** standpoint. The results are expressed in **p.p.m.** Drinking water must never be considered safe, however, without a satisfactory **bacteriologic** analysis.

CHEMICAL ANALYSIS OF MILK

Reagents

N/10 sodium hydroxide:

1. To about 50 ml. distilled water in a 100 ml. volumetric flask add 10 ml. N/1 sodium hydroxide solution.
2. Dilute to 100 ml. with distilled water.

Benedict volumetric solution:

1. The method of preparing and titrating Benedict volumetric solution is found on pp. 1813ff.

Bogg reagent for protein:

1. Phosphotungstic acid	25 gm.
Distilled water	125 ml.
2. Concentrated hydrochloric acid	25 ml.
Distilled water	125 ml.

When the phosphotungstic acid is completely dissolved, mix the 2 solutions. This reagent is quite stable if kept in a dark glass bottle.

Alcoholic phenolphthalein, 1%:

1. Dissolve 1 gm. phenolphthalein in 100 ml. 95% alcohol.

Milk-fat mixture:

1. Equal parts of concentrated hydrochloric acid and amyl alcohol.*

Sulfuric acid, 75%:

Distilled water	25 ml.
Sulfuric acid, c.p., concentrated	75 ml.

Caution: Add the sulfuric acid slowly to the distilled water in a Pyrex flask, cooling it under running tap water.

Reaction

Usually pHydrion or Nitrazine paper is sufficient. If a more detailed analysis is required, the following procedure may be used.

Procedure:

1. Make a preliminary determination of the reaction with test paper (litmus, pHydrion, or Nitrazine). If the milk is alkaline, the degree of alkalinity is determined by titration with hydrochloric acid; if it is acid, titrate with sodium hydroxide.
2. Place 10 ml. milk in a casserole.
3. Add about 50 ml. distilled water, and 2 drops 1% alcoholic phenolphthalein.
4. Add the N/10 sodium hydroxide (or HCl) drop by drop from the buret, stirring constantly, until the first permanent pink color is obtained. This is the end point.
5. Read from the buret the number of ml. N/10 sodium hydroxide (or HCl) used. Multiply this number by 10 to obtain the degree of acidity.

Example: If 1.5 ml. N/10 sodium hydroxide were used to neutralize 10 ml. milk, the degree of acidity is 10 × 1.5 or 15°. The degree of acidity is equal to the number of ml. N/10 sodium hydroxide required to neutralize 100 ml. milk. An acidity of 15-25° may be observed within 6 hr. after milking; 48 hr. after, the acidity is generally between 100 and 125. This applies more to cow's milk than to human milk.

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*Make fresh each day.

Specific gravity

Take the specific gravity with a urinometer. If a bacteriologic examination is to be made, transfer enough milk to a sterile container for that purpose before the urinometer is admitted. Normal specific gravity is 1.028-1.032. The milk should be cooled to 60° F. before taking the specific gravity.

Color

The normal color of human milk is pale bluish white.

Lactose

1. Fill a buret, graduated in 0.1 ml., with milk. Place 25 ml. Benedict volumetric solution in a casserole. Add 5-10 gm. sodium carbonate and a pinch of powdered pumice.
2. Bring the solution in the casserole to a boil and continue boiling until the end point is reached.
3. Add the milk to the Benedict solution in the casserole, a drop at a time, stirring after the addition of each drop. The milk should be added very slowly to the solution in the casserole, and some time should be allowed to pass before the addition of each drop, as reduction of the copper takes place very slowly.
4. Continue adding the milk and boiling until the blue color of the copper sulfate has entirely disappeared and is replaced by a gray color. This is the end point.
5. Read from the buret the number of ml. milk used; 25 ml. Benedict volumetric solution are reduced by 67 mg. lactose.

The number of ml. milk that were used to bring about the reduction of the copper sulfate, therefore, contained 67 mg. lactose. From this figure, the number of gm. lactose/100 ml. milk, or the percentage of lactose, can be computed according to the following example.

Example: It required 4.0 ml. milk to reduce 25 ml. Benedict volumetric solution. 4.0 ml. milk, therefore, contain 67 mg. lactose. 1 ml. milk contains 67 divided by 4, or 16.75 mg. lactose. 100 ml. milk contain 16.75 × 100, or 1675 mg. lactose. 1675 mg. = 1.675 gm. (1675 ÷ 1000). The percentage of lactose in the milk is therefore 1.675.

Normal percentage of lactose in human milk is 6-7; in cow's milk, 4.3-7.

Fat—Leffmann-Beam method

1. Use the modified Babcock tube, made for 5 ml. quantities of milk. Place 5 ml. milk in the Babcock tube.
2. Add 1 ml. milk-fat mixture. Mix thoroughly by rotating.
3. Add 75% sulfuric acid to the mixture, mixing thoroughly as the acid is added, until the meniscus stands at the 0 mark on the stem. The solution becomes quite hot and must be centrifuged before it cools.
4. Centrifuge immediately at 1000 r.p.m. for 3 min. The fat will be separated and may be read in percentage on the stem. Each mark on the stem is 0.2% of fat.
5. Read the percentage of fat from the stem and record. Read from the top of the meniscus.

Normal percentage of fat in human milk is 3-5; in cow's milk it is 3.5-4.

Protein—Bogg modification of Esbach method

1. If human milk is to be examined, dilute, accurately, 1:10 with distilled water. If cow's milk is being tested, dilute 1:20 with distilled water. Place the milk in an Esbach tube until the bottom of the meniscus stands at mark U.
2. Fill to mark R with Bogg reagent (p. 2082).
3. Mix by inverting several times, taking care to prevent the formation of air bubbles.
4. Allow to stand in an upright position for 24 hr.
5. Read from the graduation marks the number of gm. of dried protein/1000 ml. or the height of the coagulum. Divide this result by 10 to obtain the number of gm. in 100 ml. Multiply this by the dilution of the milk. This gives the number of gm. protein in 100 ml. or the percentage.

Example: Human milk was used, and the dilution was 1:10. The reading on the Esbach tube is 1.25. 1.25 ÷ 10 gives 0.125 or the number of gm. protein in 100 ml. diluted milk. 0.125 × 10 = 1.25 gm. of protein in 100 ml. milk or 1.25% protein.

Normal protein content of human milk is 1-2.25%; of cow's milk it is 1.5-4%.

Protein may also be determined from total nitrogen values. Assay a 1:5 or a 2:5 dilution of milk for nitrogen by the method described on p. 41. The protein value is obtained by multiplying the % nitrogen by 6.34.

Total solids

Total solids are roughly calculated from the lactometer readings according to the following formula:

Let L equal the last 2 figures of the specific gravity.

Let F equal the percentage of fat found in the milk.

Total solids equals:

$$(L \div 4) + (0.2 \times F) + F.$$

Example: Specific gravity was 1.028. Percentage of fat was 4. 28 ÷ 4 is 7; 0.2 × 4 (F) is 0.8; 7 + 0.8 + 4 = 11.8% total solids.

Total solids may also be determined by drying an aliquot of milk to a constant weight. The volume used multiplied by the specific gravity is the original or wet weight. The dry weight times 100 divided by the wet weight is equal to the per cent solids. In this case any volatile material is considered water.

Formaldehyde in milk

1. Place 5 ml. sulfuric acid, c.p., concentrated, in a test tube.
2. Add to it 15 drops 10% ferric chloride solution. Great caution must be taken in the addition of the ferric chloride solution to the sulfuric acid. Do not add it too rapidly and avoid undue agitation of the contents. The

proportion of ferric chloride is 3 drops to each ml. sulfuric acid.

3. Fill a large test tube to within about 1 in. of the top with the milk to be tested.
4. Allow 1 or 2 ml. sulfuric acid-ferric chloride mixture to run slowly down the side of the tube so that it will collect on the bottom of the tube without mixing with the milk.
5. Allow to stand about 5 min.

The development of a reddish ring at the point of contact indicates the presence of formaldehyde.

Care must be taken not to use too much reagent, which may obscure the reaction.

Chapter 118

Skin tests

SKIN TEST FOR DIPHTHERIA
Schick test for susceptibility to diphtheria infection[37]

Schick toxin and controls may be purchased from any reliable biological supply house. This toxin is diluted and ready for use; 0.1 ml. of this mixture is 1/50 the minimum lethal dose of diphtheria toxin. This is the quantity to be injected.

1. Disinfect the flexor surface of the forearm.
2. Withdraw the mixture in the vial using a 1 ml. tuberculin syringe with a 23-gauge ½ in. needle. The syringe should be graduated in 0.1 ml. The beveled edge of the needle should be in line with the graduations of the syringe.
3. Push the needle between the layers of the skin until the point is well covered by the skin. The injection is to be made intradermally; hence, care must be taken not to give a deep inoculation. The point of the needle can be seen under the skin if the injection is made correctly.
4. Inject 0.1 ml. of mixture between the layers of the skin. A small white wheal will form.

Saline control: About 2 in. below this test puncture, inject 0.1 ml. sterile physiologic saline.

Protein control: After the injection of the Schick toxin inject 0.1 ml. heated mixture (control) intradermally about 2 in. below the saline injection. If the reaction is not read until the third or fourth day, this last injection may be omitted. Protein reactions occur within 24 hr. and can therefore be eliminated in the final reading.

Reading the reaction: The patient is instructed to return to the laboratory every day for 4 days. If a reaction occurs within 24 hr., it usually is not a diphtheria reaction. If the reaction is due to hypersusceptibility to saline, the saline control will show a zone of inflammation and a swelling. If the reaction is due to proteins, it is much more vigorous than the true Schick reaction and will reach its height within 24-48 hr.

A **negative reaction** is indicated by absence of change in the skin.

A **positive reaction** is accompanied by swelling and a zone of inflammation around the point of inoculation. The zone of inflammation is about 1 or 2 cm. in diameter around the point of inoculation. The inflammation gradually disappears and is replaced by browning of the skin accompanied by a slight area of necrosis. The slight brown discoloration may persist for several months.

A positive Schick test is indicative of susceptibility to diphtheria infection.

A negative reaction indicates immunity to diphtheria infection.

After the toxin has been diluted with the saline, it cannot be used for a period longer than 24 hr.

SKIN TESTS FOR SCARLET FEVER
Dick test for susceptibility to scarlet fever

Scarlet fever is ordinarily streptococcal sore throat in which the infectious agent is capable of producing erythrogenic toxin, and the patient has relatively low antitoxic immunity. In 1923 Dick and Dick[38] established facts which seemed to prove that the hemolytic streptococcus is responsible for scarlet fever. They showed that filtrates of broth cultures of scarlatinal strains of *Streptococcus pyogenes* (group A) contained a toxic substance which produced a typical erythematous reaction when injected intradermally in a proportion of normal persons who had not suffered from scarlet fever. The administration of larger doses of erythrogenic toxin to susceptible persons induced a generalized reaction with fever, nausea, vomiting, and transient scarlatiniform rash.

Purchase the Dick toxin for skin tests from any reliable biological supply house. This product is made so that 0.1 ml. represents the quantity for injection.

1. Cleanse the flexor surface of the forearm with alcohol.
2. Using a 1 ml. tuberculin syringe, inject intradermally exactly 0.1 ml. Dick toxin.
3. With another syringe, inject 0.1 ml. sterile physiologic salt solution about 2 in. below the test injection. Needles should be the same as for the Schick test.
4. Wait 24 hr.
5. Read the reaction in a good light.

If the saline injection shows a reaction, disregard the result of the test.

If the saline injection shows no reddening, the test may be read.

The readings are as follows:

Negative reaction: Red area less than 1 cm. in diameter.

Slight positive reaction: Red area 1-2 cm. in diameter.

Positive reaction: Red area 2-3 cm. in diameter, with some swelling and tenderness.

Strong positive reaction: Red area more than 3 cm. in diameter that is swollen and tender.

Blanching test for diagnosis of scarlet fever

In 1918 Schultz and Charlton[39] reported that 1 ml. normal human serum injected into skin of a scarlet fever patient produced blanching of the rash in the immediate neighborhood of the injection. Mair[40] viewed the positive reaction as the result of the development of a specific antitoxin which neutralizes the toxin present in the skin of a scarlet fever patient.

The test is now performed with 0.5 ml. of a good convalescent serum or 0.1 ml. of a potent antitoxin, and fading of the rash can be expected in 6-12 hr. A positive reaction establishes the diagnosis of scarlet fever and is useful in differentiating the scarlatinal rash from similar rashes caused by German measles and certain drug idiosyncrasies.

TUBERCULIN SKIN TESTS

Scratch test

Koch's old tuberculin (OT) may be obtained from any reliable biological supply house.

1. Cleanse the patient's forearm with alcohol.
2. Make 3 scratches (superficial) in the skin, each scratch about ½ in. in length, and at a distance of about ½ in. from each other.
3. Into the first 2 scratches (test) rub a small amount of tuberculin, using a sterile matchstick.
4. Into the third scratch rub a small amount of sterile physiologic salt solution (control).
5. Allow these to dry.
6. Instruct the patient to return to the laboratory for examination at the end of 24, 48, and 72 hr.

If the reaction takes place within 24 hr. he need not repeat the visits.

A positive reaction is characterized by a slight swelling along the scratch and an area of inflammation surrounding the inoculation.

The saline control should show no swelling or redness. If there is a reaction due to saline, the result of the test is unreliable.

A negative reaction shows no change in the skin.

A positive reaction persists for several days.

Koch subcutaneous tuberculin test

In this method Koch's OT is used. This is a filtrate of a concentrated broth culture of tubercle bacilli containing the toxin of the bacteria. It produces a focal as well as a general reaction. Before applying the tuberculin test take the temperature of the patient for several days to determine variations. Contraindications to the test are a markedly febrile course, general miliary tuberculosis, acute or advanced tuberculosis, tuberculous meningitis, the terminal stages of other diseases, and during convalescence from acute febrile diseases. The usual dose is 0.5 mg. injected subcutaneously.

A positive reaction consists in an elevation of temperature of at least 1° F., focal reaction, and a local reaction at the site of the injection. If no reaction is produced, repeat the injections, increasing the dosage gradually every 3-5 days until as much as 5 mg. have been given.

Mantoux intradermal tuberculin test

In this test a measured amount of OT is injected between the layers of the skin. For this test the following solutions are needed: 5 solutions of OT—1:10, 1:100, 1:1000, 1:10,000, and 1:20,000. They may be purchased ready for use, and if kept in a cool place, they will remain potent for 3 years.

1. Sterilize a 1 ml. tuberculin syringe and several 26-gauge ½ in. hypodermic needles.
2. Sponge the forearm with alcohol.
3. Fit a needle to the tuberculin syringe, with the bevel of the needle on the same side as the graduations on the syringe.
4. Fill the syringe to 0.2 mark with the OT.
5. Pinch the skin and press in the needle until the lumen just disappears. The injection raises a white wheal.

In patients who have had recent hemoptysis or skin or joint disease, the initial dosage should be 0.1 ml. 1:10,000 solution.

Read the reaction on the second to fourth day. An area of erythema ⅜ in. or more in diameter is positive. The more marked reactions show swelling and at times necrosis. When the reaction is negative, repeat the test, using 0.1 ml. 1:1000 dilution, and if still negative 48 hr. later, repeat, using 0.1 ml. 1:100 dilution, etc.

Vollmer tuberculin patch test*

This is a quick, accurate, painless test obviating the use of instruments as well as injury to the skin.

1. Thoroughly cleanse and defat with acetone the area of the skin of the inner side of the forearm or sternum (recommended for children) or upper spine (recommended for infants).
2. Then apply the patch firmly. Instruct the patient not to disturb the patch for 48 hr. and to avoid getting it wet.
3. The second day after removal, have the patient return to the laboratory for interpretation of the test.

A **positive** reaction appears as an infiltrated elevated reddened square. The central control square appears pale. If the patient is sensitive to adhesive tape, there may be irritation due to the tape, but this does not interfere with reading of the reaction.

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*Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

If directions for making and reading the test are carefully carried out, the results will be found superior to the von Pirquet test and comparable to those of the Mantoux test. Late reactions should be reported to the physician.

Rosenthal tine test*

This test is a simple rapid procedure. It correlates closely with the Mantoux test (94.3% for positive reactions and 96.8% for negative reactions).[41, 42]

The tine test is an intradermal test for the detection of tuberculin sensitivity. It is standardized by comparison with the standard intradermal Mantoux test (5 ITU).

Each tine test unit consists of a stainless steel disk attached to a plastic handle. Projecting from the disk are 4 triangular-shaped prongs, or tines, which are 2 mm. long and approximately 4 mm. apart. The tines have been dip-dried into 4 times concentrated U.S. Standard OT (U.S. Standard OT is equivalent to International Standard OT). The unit is disposable, thereby eliminating the need for syringes, needles, and other equipment for tuberculin testing. The package is terminally sterilized by ethylene oxide gas, and each unit is sterile until removed from its individual holder.

The preferred site for accurate and standardized tuberculin testing is the volar surface of the mid-forearm. The skin must be clean and dry. It may be cleansed with alcohol, acetone, ether, or soap and water. Hairy areas and areas without adequate subcutaneous tissue, such as concavities over a tendon or bone, should be avoided.

Grasp the patient's arm firmly with one hand, stretching the skin of the forearm tightly, and apply the time test with the other hand. Momentary stinging may cause the patient to jerk his arm, resulting in scratching. Hold momentarily (1 sec.) and withdraw. Sufficient pressure should be exerted so that the 4 puncture sites and a circular depression of the skin from the plastic base are visible. **Do not reuse the tine test unit.**

Tests should be read at 48-72 hr. **Induration** is the only criterion. Erythema is ignored. It is important to measure the amount of induration exactly by examining under adequate light and by thoroughly palpating the area. Identification of the application site is usually easy because of the distinct 4-point pattern. Two mm. or more of palpable induration around one or more puncture sites is a positive reaction and is equivalent to 5 mm. or more by the standard intradermal (5 tuberculin units or 0.0001 mg. PPD) Mantoux test. With the more positive reaction and is equivalent to 5 mm. or more puncture sites tend to coalesce.

Tuberculin testing should be done with caution in persons with active tuberculosis. However, activation of quiescent lesions is not to be expected.

Interpretation of tuberculin reactions

The tuberculin reaction is a reliable test for hypersensitiveness to the tubercle bacillus; by that we mean past or present tuberculous infection. The negative reaction is of most value in clinical diagnosis. When tuberculosis nears fatal termination, sensitiveness to the tuberculin injection is depressed.

In children, a negative reaction is useful to exclude tuberculosis in chronic cervical adenitis, prolonged anorexia, debility following bronchopneumonia, whooping cough, or measles. If complete healing of a tuberculous infection occurs and the bacilli die, the tuberculin reaction after a time may become negative. Sometimes, infection becomes well walled off, often in a calcified nodule, but still the bacteria remain alive, and a positive tuberculin reaction is noted. A markedly positive Mantoux reaction in a patient with indefinite physical signs suggests some active focal infection which under unfavorable hygienic conditions may spread and form extensive disease.

SKIN TESTS FOR LYMPHOGRANULOMA INGUINALE (VENEREUM)

Frei reaction for lymphogranuloma inguinale (venereum)

This is a method of making the diagnosis of lymphogranuloma inguinale (venereum) by the use of an antigen prepared from the pus withdrawn by aspiration from the bubo. It was published by Frei[43] in 1925. The value and specificity of the test have been demonstrated by Frei in subsequent papers[44-46] in 1927 and 1932 and have been confirmed by Frei and Hoffmann[47] in 1927 and by a number of other workers.

Frei's directions for the preparation of this antigen are as follows: The person from whom the antigen is to be made should be proved free from tuberculosis and venereal diseases by clinical examination and negative tests for syphilis, gonorrhea, and soft chancre. The pus must be withdrawn from a gland that has undergone softening but not fistulation. Aspirate by means of syringe and thick needle, under sterile precautions, obtaining as far as possible blood-free pus. This pus is mixed with physiologic saline in the proportion of 1 part pus to 5 parts saline in a sterile tube and is immediately put up in 0.5-1 ml. doses in hard-glass ampules. It is heated to 60° C. for 2 hr. over a water bath and the following day at 60° C. for 1 hr. This material is tested for sterility by culture under aerobic and anaerobic conditions at room and incubator temperatures. This antigen should give a negative reaction in the skin of normal individuals. The antigen should be preserved at a low temperature and should not be exposed to light. If an ampule is opened, the remaining contents should be discarded or resterilized.

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*Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y.

The method of carrying out the test is as follows. Disinfect the skin with alcohol. Use the skin of the outer forearm. Inject intracutaneously 0.1 ml. antigen. A corresponding area on the other side of the body should be injected with the same amount of physiologic saline. It is well to inject 2 normal controls with the same antigen and the same saline in the same way and at the same time. Read the result after 48 hr. and not before. A positive reaction consists in the production of an infiltrated inflammatory dome-shaped area 0.5 cm. in diameter at least and often larger, which can be felt as well as seen. It is often noted that there is a small area of necrosis in the center surrounded by a red zone.

Antigens usually remain active for several months, sometimes for a year, but not always.

Vesicular test for lymphogranuloma inguinale (venereum)

Because of the difficulty in obtaining material at times for making the original Frei antigen, Ottolina[48] suggested the use of concentrated spinal fluid from a known case, using this material for skin inoculation. This is based upon the fact that in experimental infections of this disease the cerebromedullar axis is prevailingly affected. Spinal fluid, therefore, constitutes a human antigen, easy to obtain, aseptic, and with a small amount of albumin.

The method described by Ottolina is to perform a spinal puncture under aseptic conditions and carry out a vacuum concentration of the fluid. By reducing 10 ml. fluid to 1.5 ml., a colloidal-like solution is obtained which is opalescent and foams upon agitation. If this concentrated fluid is injected intradermally, a vesicle appears 24 hr. later at the point of injection; this phenomenon has been called the "vesicular test." The vesicle may be ovoid, circular, or triangular, but there is a definite trend toward the hemispheric form. The ovoid type measures 4 mm. in length, 2 mm. in width, and 1 mm. in height, whereas the more rounded forms approach approximately 3 mm. × 2 mm. × 1 mm. The color of the skin covering the blister is light or dark brown, sometimes dark gray. The blistered skin always has a darker shade than the surrounding skin.

Twenty-four hr. after the injection the blister preserves the same morphology, the fluid within showing a light tension. Forty-eight hr. later a certain umbilication may be noted, the fluid tension has relaxed, and the blister skin is more colored than on the first day. Three days later there is a distinct flaccidity in the blister and desiccation begins at that time and ends in 4 or 5 days. Five or 6 days after its appearance the blister is only a pigmented spot about 2 mm. in diameter. The vesicular fluid has a light brown color and microscopically shows a few polynuclear neutrophilic and mononuclear leukocytes. This fluid is sterile.

Technic is to concentrate fluid employed from 10 ml. to 2 ml. and inject intradermally 0.3 ml.

It is interesting to note, according to Ottolina's researches, that this test, when applied to the positive patient from whom it was obtained, produced a typical positive reaction. In other words, it is an autoreaction, which emphasizes its diagnostic value.

Grace-Suskind mouse brain antigen* for Frei reaction

Hellerström[49] has shown that antigens made from the brains of monkeys infected through subdural injection give the same specific Frei reaction when used on human beings as when the human antigen is used. Working along this line, Grace and Suskind[50] developed a mouse brain antigen which is more highly potent than that made directly from the same human material used in the preparation of the brain antigen. This antigen is a 10% emulsion of infected mouse brain.

For each test inject 0.1 ml. intradermally, using a 1 ml. tuberculin type of syringe with a 27-gauge needle. If **positive,** the raised papular reaction is noted in from 48-72 hr. If **negative,** no reaction occurs; if one uses an injection of a similar emulsion of normal mouse brain as a control, no reaction appears.

Chick embryo antigen† for Frei reaction

The 3 sources of antigen for the Frei test just described are (1) pus from the inguinal bubo of the patient, (2) spinal fluid from a patient with the disease, and (3) emulsions of the brains of mice intracerebrally infected with virus. Since antigens prepared from human pus and spinal fluid are not readily available, and since considerable doubt has been expressed as to the reliability of the mouse brain antigen, a successful method of producing antigen has been evolved by propagating the virus of lymphogranuloma inguinale on the chorioallantois of developing chicks. This has been successfully carried out by Rake et al.[51] working in the Squibb Institute of Medical Research. These workers developed a method for obtaining a highly potent and purified antigen for this test. The virus was used to inoculate into the yolk sac of 5- or 6-day-old fertile eggs, as demonstrated by Fig. 794.

By suitable methods of purification a virus was obtained which, when undiluted, was about 1 million times more potent than the lymphogranuloma virus obtained from mouse brain. Not only did the yolksac method yield a more potent virus than the other methods,

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*Lederle Laboratories, Pearl River, N. Y.

†E. R. Squibb & Sons, New York, N. Y.

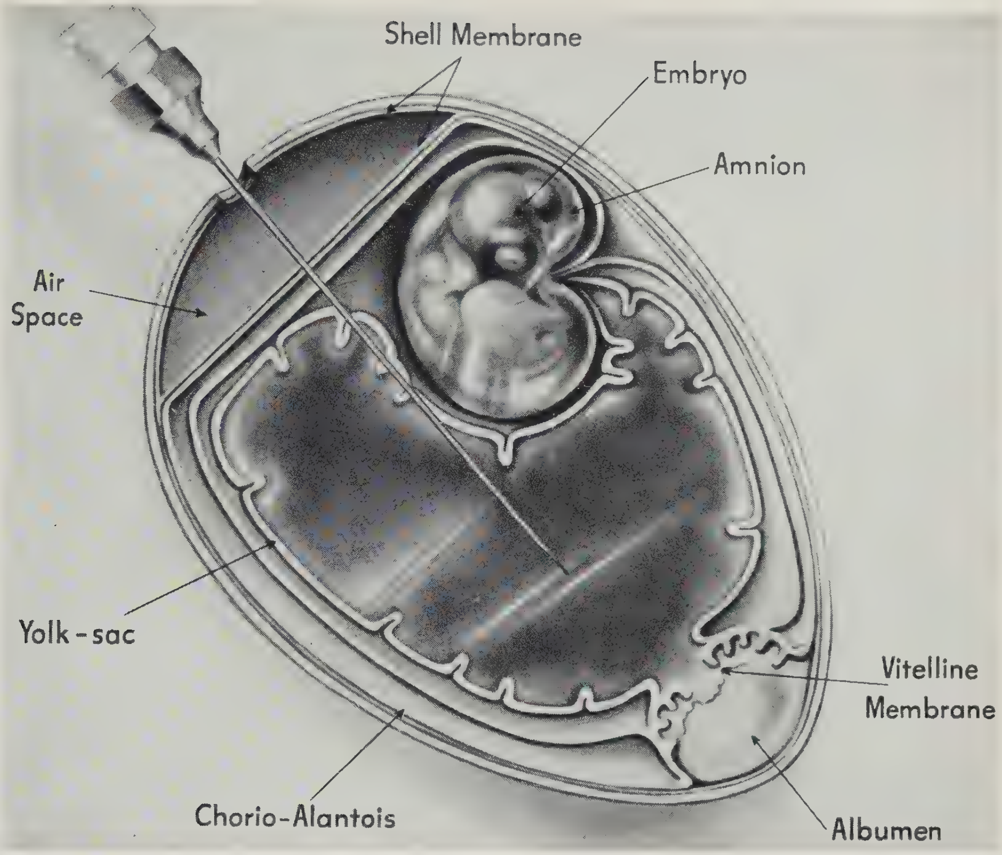

Fig. 794. Method of injecting the yolk sac of a developing chick. (Courtesy E. R. Squibb & Sons, New York, N. Y.)

but also by a process of differential centrifugation, a practically pure suspension of the virus could be obtained. In other words, the extraneous inert material from the yolk sac can be almost completely eliminated.

In a very interesting trial of this material, Sulkin et al.[52] reported 42 patients with active lymphogranulomatous infection or with a history of past infection who were tested with the various antigens. They made a series of intradermal injections on the arm, the proximal injection being made with human Frei antigen, 2 middle injections with egg and mouse brain antigens, and 2 distal injections with the corresponding controls. Reactions were read within 48-96 hr. Of the 42 patients tested, 40 gave a positive, 1 a doubtful and 1 a negative reaction to yolk sac antigen; 34 gave a positive, 5 a doubtful, and 3 a negative reaction to mouse brain antigen; 38 gave positive, 1 a doubtful and 3 negative reactions to human pus antigen. Thirty-three persons had positive reactions with all three antigens.

As these writers point out, any foreign substance when injected intradermally into a human subject will produce an inflammatory response at the site of inoculation, and the severity of the reaction will be influenced by any hypersensitivity to the material. These facts, of course, must be considered in determining the cutaneous response to any Frei antigen. The conclusions of these workers, however, were that the yolk sac antigen is superior to mouse brain antigen for performance of the Frei cutaneous test for lymphogranuloma inguinale and that comparatively few nonspecific reactions are produced with the egg control material as compared with the mouse brain control.

Procedure of the test: Inject 0.1 ml. Frei antigen of chick embryo origin intradermally into the flexor surface of the arm. Read reaction within 48 or 72 hr. In order to eliminate false positives due to chick protein sensitivity, a control is supplied by the manufacturer for each test. This consists of normal chick embryo protein prepared in a manner similar to that of the antigen. A positive test is seen by the appearance of a reddish papule 6 mm. or more in diameter, surrounded by a fainter erythematous area of varying size. This papule must be measured with a millimeter scale. The extent and intensity of the erythematous area around the papule is of no diagnostic significance. Strongly positive reactions may have a papule surmounted by a vesicle, or pustule which may break down and form a crust. The reaction will gradually regress, ending as a faintly pigmented area with or without a scar.

The intradermal Frei test is positive only at the peak of the allergic period when specific antibodies have been formed in the infected organism. This usually becomes positive about 2 wk. after inoculation, sometimes later. It may be negative during the menstrual period and in the not-so-rare cases of florid syphilis if the 2 diseases coexist.

Interpretation of results should be made from the fourth day. The reaction appears in 24-48 hr., reaching a maximum in 3 days, after which it gradually decreases in intensity. A positive reaction consists of a papule, vesicle, or pustule with a diameter larger than 6 mm. It is more intense in inguinal than in rectal cases. The reaction persists for several weeks. Saline control injection should always be made. Nonspecific reactions have a diameter of less than 6 mm. and decrease after 3 days.

Most of the American workers agree that the chick embryo antigen is superior to all others for use in the Frei test.

The sole disadvantage of this test is its delayed appearance. Ravaut et al.[53] proposed a hemoreaction for early diagnosis. Their antigen is injected intravenously, producing a rise of temperature to 102°-104° F. in 12-18 hr. which remains for 24-48 hr. and is accompanied by general malaise, chills, headache, insomnia, weakness, arterial hypotension, and leukopenia. The test has the disadvantage that it can be used only once. It is positive in 97% of the cases.

The complement fixation test may give false positive results, especially in diseases caused by viruses belonging to the same group.

The presence of Miyagawa corpuscles in infected tissues as seen in secretions is considered diagnostic of the disease. These corpuscles can be demonstrated with certain stains: eosin and methylene blue, Macchiavello, Giemsa, and Noble.

The blood picture shows leukocytosis, oligocytosis, neutrophilia, lymphopenia, and monocytopenia.

SKIN TESTS FOR CHANCROID

Greenblatt-Sanderson intradermal chancroid test

In connection with the foregoing remarks on lymphogranuloma inguinale, it must be remembered that every case of venereal disease requires exhaustive laboratory tests to prove the true diagnosis. It has been found that many cases of chancroid infection have been thought to be lymphogranuloma inguinale but a negative Frei test has caused some doubt concerning this diagnosis. Greenblatt and Sanderson[54] have called attention to a very valuable method of including or excluding chancroid infection in a diagnostic survey of these venereal cases. They have originated an intradermal test for chancroid infection using an antigen made from a saline suspension of *Haemophilus ducreyi* bacillus. Their method of growing this bacillus is as follows.

Method of cultivating Haemophilus ducreyi: To tubes of beef infusion agar in slants is added 1 ml. defibrinated human blood. The blood is inoculated with 0.1 ml. from an actively growing culture and placed under partial oxygen tension. To obtain this environment, the cotton plug is cut off short and pushed slightly into the tube; the upper portion of the tube is heated, but not too much, in a Bunsen flame, and the tube is sealed by inserting a tightly fitting rubber stopper. The culture is incubated for 2-3 days at 37° C. During this period good growth takes place in the fluid blood, and a lesser growth appears on the surface of the slant. After the incubation, the blood, as well as the surface growth, if any, is removed with a sterile pipet and added to approximately 25 ml. sterile distilled water in a sterile centrifuge tube of sufficient volume, and the contents are sedimented by high speed centrifugation. The supernatant fluid is removed aseptically and discarded, and the process is repeated. Two such treatments suffice to remove most of the hemoglobin. The sediment of bacilli and red cell debris is suspended in sterile physiologic solution of sodium chloride, 10 ml./slant being placed in sterile vials, and heated for 30 min. at 60° C. A 1:10,000 solution of Merthiolate is used as a preservative. One-tenth ml. is employed for intracutaneous tests, and the reactions are observed in 48 hr.

It must be remembered that some patients with lymphogranuloma inguinale with a positive Frei test are also suffering from a chancroid infection, in which case the intradermal chancroid reaction will be positive. A positive chancroid test is only presumptive evidence and does not necessarily mean that the lesion under consideration is chancroid. A similar conclusion should be borne in mind in the interpretation of a reactive Wassermann as well as of a positive Frei test in every case of genital lesion unless other confirmatory laboratory evidences are present. A negative intradermal test with bacillary antigen is diagnostic so far as it rules out infection by the bacillus of Ducrey. A positive intradermal chancroid test commits one to the diagnosis of chancroid infection only when the Frei test, standard tests for syphilis, and microscopic examinations for malignant disease and granuloma inguinale give negative results. This reaction remains positive for life, and this fact should be borne in mind in dealing with the above considerations.

SKIN TESTS FOR BRUCELLOSIS

Brucella skin test*

As a consequence of the infection by any of the 3 brucellae, the host develops a state of hypersensitivity displayed after further exposure to these organisms, whether live or killed. The injection by intradermal route of small amounts of a suspension of inactivated brucellae or soluble antigens is usually followed by a delayed tuberculin-like dermal reaction which may reach a peak 24-48 hr. after injection. The reaction may last several days and in some cases may be of such a high intensity as to produce local necrosis accompanied by regional lymphatic reaction.

The antigens most commonly used have been heat-killed *Brucella* cells suspended in isotonic saline, broth filtrates known as **melitin or abortin,** certain purified products such as the protein antigen (PPA) of Moreles-Otero, the brucellergen of Huddleson, and the polyvalent antigen (MBP) prepared at the Brucella Center of Mexico. The test is performed by intradermal injection into the forearm of 0.1 ml. of any of the above mentioned antigens.

It is advisable to use antigens that are not capable of stimulating agglutinin response in nonreacting patients.

Since the skin test is likely to be positive not only in active cases of brucellosis but also in any person previously exposed to the infection, one has to be aware of the inconvenience of using this procedure as diagnostic evidence of actual infection. Furthermore, it has been observed that the skin test is not always positive during the evolution of clinical brucellosis. It is expected that the test will be negative during the first weeks of illness, but even in the third month there may be as many as 48% negative tests in bacteremic cases. The percentage of negative tests drops to 31 in the sixth month and to 23 from the sixth to the twelfth month. In nonbacteremic cases the test is generally positive, particularly in chronic cases.

Because of the difficulty of properly interpreting the skin test, one must consider it as an additional evidence of infection together with other tests. It is occasionally of help in deciding doubtful cases with symptoms compatible with brucellosis but with low agglutinin titers and negative blood cultures. However, the final decision must await further evidence. On the other hand, a persistently negative skin test is of value in ruling out brucellosis in patients with chronic illness.

By means of carefully standardized allergens such as the polyvalent MBP, it has been observed in epidemiologic surveys that reacting persons living in endemic areas, healthy at the time of the test, have shown reactions of moderate intensity. If after treatment with such antigen a patient with a known history of brucellosis or suggestive symptoms develops reactions of unusual intensity with or without a systemic response, he may be considered a hypersensitive individual in whom the allergic state might be related to the actual clinical condition. Those who favor allergic desensitization may find the allergic test of some value as a guide in therapeutic attempts.

SKIN TESTS FOR HANSEN'S DISEASE

Lepromin test (Mitsuda test)

This test consists essentially of an intradermal injection of lepromin, an antigen prepared from tissues rich in Hansen bacilli. It is made by boiling lepromatous tissues in isotonic solution of sodium chloride for 1 hr., after which the mass is ground in a mortar, and 20 ml. 0.9% solution of sodium chloride are added for each gm. of ground tissue. After repeated and thorough mixing and grinding, the supernatant fluid is pipetted off, filtered through gauze, and stored in a sterile container.[55]

The liquid is autoclaved at 120° C. for 15 min., 0.5% phenol is added, and the liquid distributed to rubber-capped vials ready for use. It is a cloudy, milky emulsion containing numerous bacilli (globi) and the tissue elements of the leproma. If kept in a dark, cool place, it retains its activity for a long time. Seitz-filtered antigens are inactive.

Procedure:

Inject 0.1-0.15 ml. lepromin intradermally either into the skin of the scapular region or upon the anterior surface of the thigh.

At the end of 24-48 hr. there is a light erythema which is attributed to the presence of foreign protein material and which disappears in a few days, usually in 3 or 4. The site of the test then remains apparently normal until the beginning of the third week when, if the test is positive, a slight papular infiltration is noted, gradually increasing in size until it attains the size of a pea. The infiltration is deep, and its color is bright red. There might be some tenderness on pressure and occasionally formation of a central necrotic focus with ulceration, resulting in a permanent scar.

The test should be evaluated on about the thirtieth day after the injection. If there is no reaction or only a small (less than 5 mm.) infiltration, the test is negative.

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*M. Ruiz Castañeda.

The test is positive in about 90% of normal individuals, in practically all cases of tuberculoid Hansen's disease, and in about 50% of patients with the simple inflammatory type. It is negative in practically every case of lepromatous Hansen's disease, in about 10% of apparently normal persons, and in about 50% of patients with simple inflammatory Hansen's disease.

It is a **prognostic test.** If the test is negative in a person without symptoms of Hansen's disease, that person is susceptible to the disease. The test is applied to prospective employees of leprosaria; those with negative Mitsuda tests are not permitted to serve in such institutions.

Histamine test

1. Place a drop of 1:1000 solution histamine phosphate on the affected skin to be tested.
2. Place another drop at a point between the suspected area and the adjacent normal skin and a third drop on the normal skin.
3. Lightly prick the epidermis through these drops, using the point of a hypodermic needle, being careful not to cause bleeding.
4. Immediately dry the drops of histamine with cotton and carefully watch the sites of the test.
5. Within 25-40 sec. the **normal skin** shows an area of erythema with irregular outline, usually 5-20 mm. in diameter, later reaching as much as 5 cm.

In 60-100 sec. a wheal forms in the erythematous area, centered by the point where the needle entered the epidermis. After 3 min. a hemorrhagic point may appear at the site of the needle prick. The wheal usually measures 2-5 mm. in diameter.

The reaction persists for approximately 20 min., but at times the erythema and the wheal are still present after 45 min. There are individual variations due to age, room temperature, and local circulatory conditions. The test cannot be made on the skin of Negroes.

Between the affected and the normal skin, the test shows the same succession of phenomena on the normal half of the skin, but on the affected half the erythematous reaction stops abruptly and is absent on that part of the skin, but the wheal is always present without the peripheral erythema.

On the **anesthetic skin,** erythema is wholly lacking, whereas the wheal is present and remains the usual length of time.

The test causes no subjective sensations, either on the normal or the affected skin.

The test is read as normal or abnormal and not as positive or negative.

Any type of degenerative peripheral neuritis may give an abnormal histamine test, and therefore it is not exclusively diagnostic of Hansen's disease. The test is not reliable when performed at a room temperature below 24° C. or when performed on aged persons with slow or impaired peripheral circulation. It is important in the differential diagnosis between Hansen's disease and syringomyelia since in the latter condition the histamine test is normal.

SKIN TEST FOR GLANDERS

Mallein test

A modification of the mallein skin test, permitting its use in human beings, is a valuable aid in the diagnosis of glanders.

One-tenth (0.1) ml. commercial mallein in a dilution of 1:10,000 is injected intradermally into the skin of the forearm. The test is read at 24 and 48 hr. Specific positive tests reach maximum erythema, 10-20 mm. in diameter, after about 18-24 hr. and fade only slightly if at all by the end of 48 hr.

SKIN TEST FOR TULAREMIA

Foshay intradermal test

A successful intradermal reaction was reported by Foshay.[56] This reaction has the appearance of a positive tuberculin reaction. It shows up in about 48 hr. Foshay test solution is made from virulent strains of *Pasteurella tularensis.* This suspension is oxidized for about 5 hr. with nitrous acid and then adjusted to a turbidity equivalent to 100 p.p.m. of Fuller's earth. The standard test dosage of this suspension is 0.05 ml. The proper time to read the response is at the forty-eighth hour after administration. Hypersensitivity of the skin is a notable feature of tularemia; therefore, caution must be used in selecting an antigen that affects the toxicity of heat-killed organisms for skin testing.

SKIN TESTS IN FUNGAL INFECTIONS

See discussion on mycology.

SKIN TESTS IN PARASITIC DISEASES

See discussion on parasitology.

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